Changes in hormone receptors and proliferation markers in breast cancers treated with neoadjuvant letrozole and the relationship with response

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To my parents, for their love, support and encouragement

To Al, for being the one I laugh with, live for and love

To David and Robbie, for their smiles

There are no secrets to success: don't waste time looking for them. Success is the result of perfection, hard work, learning from failure, loyalty to those for whom you work, and persistence.

Colin Powell (1989)

I confirm that this thesis was composed by me.

I acknowledge that the work reported was done by me in the laboratories of the Edinburgh Breast Unit at the Western General Hospital where I worked from 1st August 2001- 6th October 2003. Exceptions to this are the MIB scoring which was done together with Miss Sharon White, Professor William Miller and Professor Tom Anderson; FISH analysis of the HER2 2+ cases which was performed by Margaret Hills in the Dept of Academic Biochemistry in the Royal Marsden Hospital in London; and statistical analysis which was done with Professor Robin Prescott and Dr Linda Williams of the Department of Statistics, Edinburgh University.

This thesis has not been submitted in canditure for any other degree, postgraduate diploma or professional qualification.

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Abstract

The aim of this study was to further characterise the clinical response to primary systemic endocrine therapy in breast cancer and to determine whether it is possible to identify biological markers of tumour phenotype that can be used to predict subsequent clinical response to neoadjuvant treatment with letrozole for three months.

137 postmenopausal patients with locally advanced oestrogen receptor (ER) positive breast cancer were treated with 2.5mg letrozole daily. Tumour samples were taken at diagnosis, at three months and, in 62 patients, additionally at 10-14 days. Serial clinical tumour measurements were made over the three month treatment period. Ultrasound scanning (USS) was shown to be the most accurate method of assessing clinical response to treatment and the modality that corresponded most closely with pathological response. Patients with ER rich tumours were shown to be those most likely to derive maximal benefit from neoadjuvant letrozole.

In this series, 67% of patients showed a clinical response to treatment (> 50% reduction in tumour volume at three months on USS) and 63 % had their surgery down-staged from mastectomy to breast conserving surgery. Of the 125 patients who completed the 3 month audit period, with a mean follow up period of 39 months (4-58), 42 patients had died. Of these, 16 had evidence of recurrent breast cancer at the time of death. 7 local recurrences have occurred in the series (5%).

75% of tumours displayed evidence of a pathological response (decreased cellularity/ increased fibrosis) at three months. Significant decreases in PgR expression were seen after both 14 days and three months but this did not correlate with clinical or pathological tumour response. Baseline proliferation, assessed using Ki67) was similar in responders and non-responders whether assessed clinically or pathologically. Treatment was associated with highly significant decreases in Ki67 in all tumour subgroups (at least $P<0.005$ by paired Wilcoxon rank test) at 14 days. There was no significant difference in Ki67 expression at 14 days between subsequent clinical responders and non-responders. However, when correlating the decrease in proliferation with pathological response, Ki67 expression at 14 days was significantly higher in tumours which subsequently failed to show morphological evidence of response.

The percentage reduction in Ki67 over the three month treatment period showed a significant correlation with cause specific survival ($p=0.007$). However, it was not possible to use changes in proliferation to predict response for an individual patient. The fresh tissue collected in parallel with the formalin fixed tissue in this study is currently being analysed by microarray and will hopefully suggest possible avenues for other markers which may prove more helpful in predicting response to neoadjuvant endocrine treatment on an individual patient basis.

Section 1: Introduction

1.1 Incidence and prevalence of breast cancer

In the western world, breast cancer is the commonest cancer to affect women accounting for a quarter of all female cancers. Approximately one in nine women in the UK will develop it during their lifetime [1].

Scotland has one of the highest incidence rates of breast cancer with an annual incidence of approximately 3,600 new cases per year (110-120 per 100,000 women per year) [2]. It results in 19% of cancer deaths in women.

Worldwide, breast cancer affects approximately one million new patients annually, causing substantial morbidity and mortality [3]. Prevalence is about five times higher. It is the third most common cancer worldwide and is responsible for 10% of the global cancer burden [4]. It principally affects women although approximately 1% of cases occur in men [5].

In Scotland, the incidence of breast cancer has risen by 46% between 1981 and 2000 (figure 1), but this has been accompanied by improved survival over recent years (figure 2).

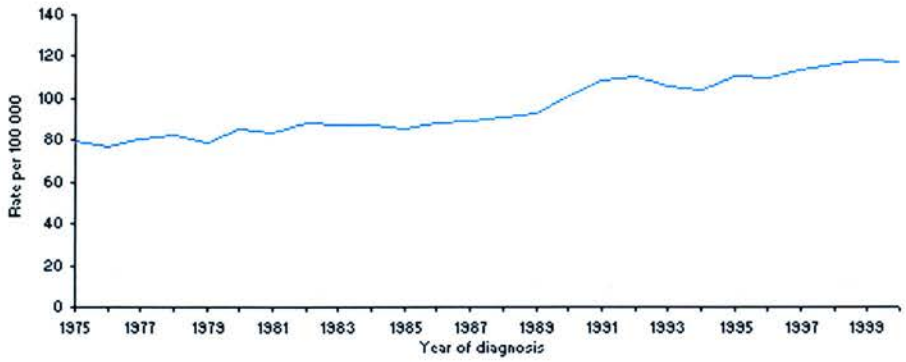

Figure 1: Trends in incidence of breast cancer in Scotland: Age-standardised incidence rates per 100 000 person-years at risk (European standard population): period 1975-2000

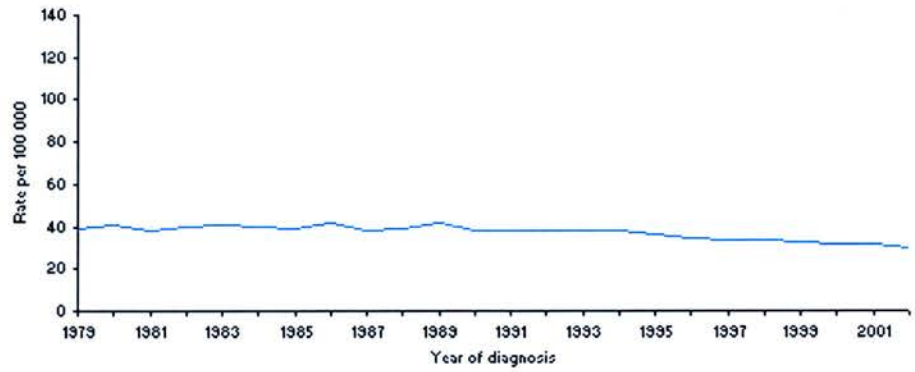

Figure 2: Trends in mortality from breast cancer in Scotland: Age-standardised incidence rates per 100 000 person-years at risk (European standard population): period 1979-2002

Mortality rates peaked in the mid 1980s and have been in steady decline since; standing at 30 per 100,000 women in 2002 [1].

The increase in breast cancer is thought to be due to a number of factors including greater longevity (one in three women over the age of 75 will develop breast cancer), women delaying childbearing until later in life and greater use of hormone replacement therapy (HRT). It may also be in part due to an increase in detection rates following the introduction of the National Screening Programme in the UK in 1988.

1.2 Aetiology

The causes of breast cancer are not fully understood. Breast cancer is unusual in women under 25 and then incidence doubles every 10 years until the menopause after which the rate of increase slows dramatically. In the UK, the average age at diagnosis is 58 and one third of all breast cancer occurs in women over 75 [6].

There is a marked geographical variation in the incidence of breast cancer between countries. Age-adjusted incidence and mortality varies by a factor of up to five between different regions. Western countries have a much higher incidence than far eastern countries although the scale of this difference is decreasing. Immigrants from countries which have a low incidence of breast cancer to countries with a higher incidence increase their own incidence to almost that of their adopted country within three generations. This suggests that environmental factors may be more important aetiological factors than genetic ones.

Breast cancer is more common in women of higher socio-economic group [7] and this may be partly due to better nutrition in early life.

1.2.1 Genetic predisposition

5-10% of breast cancers occur in women with a genetic predisposition [8]. This is most commonly inherited as autosomal dominant susceptibility genes. Two of these, BRCA1 and BRCA2, are located on the long arms of chromosomes 17 and 13 respectively. Together these genes account for a substantial proportion of high risk families. These can be transmitted through either maternal or paternal lines. Women with a BRCA1/2 mutation have a 65-85% cumulative lifetime risk of developing invasive breast cancer [8].

The main characteristics of inherited breast cancer are (i) early age of onset, (ii) two or more affected first degree relatives (especially if premenopausal at time of onset) and (iii) bilateral or multiple primaries in the same patient. In the UK, women considered to be at risk are often referred to a genetics service. Here they can discuss individual risk factors and undergo BRCA1 or BRCA2 testing if this is considered appropriate. Since both genes are very large and mutations can occur at any point, screening for a particular mutation can be difficult and time consuming.

There are certain inherited syndromes (eg. Li Fraumeni), which confer a high risk of developing breast cancer. There are also some families which have high rates of other cancers such as ovarian, prostatic or colorectal carcinoma in addition to breast cancer. These are all likely to be due to inherited mutations. There are also likely to be other as yet unidentified breast cancer susceptibility genes which increase the risk of developing

breast cancer to a lesser degree than BRCA1 and 2. These are more difficult to identify but are likely to be much commoner and may account for a significant proportion of breast cancer cases in the community.

1.2.2 Hormonal risk factors

Increased risk of breast cancer is also associated with increased length of exposure to hormonal menstrual cycling. Women who have an oophrectomy before the age of 35 reduce their risk of breast cancer by more than 40% when compared to women who have a normal menopause [9]. A woman who has a natural menopause after the age of 55 has double the risk of breast cancer compared with a woman who has her menopause before 45 [9].

Nulliparity increases the risk of breast cancer as does increasing age at first pregnancy. A young age at the birth of a second child further reduces risk. Interestingly, women who have their first child when over the age of 35 have a higher risk than those who have no children at all [9]. Breastfeeding is thought to slightly reduce the risk of breast cancer [10].

Long term use of hormone replacement therapy (HRT) and the oral contraceptive pill (OCP) (>10 years) are also thought to be associated with an increased risk of breast cancer. The risk with the OCP (1.24 relative risk) seems to disappear within 10 years of stopping treatment [11].

1.2.3 Effect of hormone replacement therapy

Over the past few years, there has been much controversy over the impact of HRT on the incidence of breast cancer and the risk of death from breast cancer. Currently, a significant proportion of women in their 50s and 60s are using HRT. Theoretically, increasing exposure to oestrogen in this way should increase the incidence of breast cancer but several early studies did not support this. However, these studies involved retrospective analysis, they had too small numbers, only had baseline information about HRT and involved many preparations that are no longer in current use.

The use of oestrogen alone or combined oestrogen and progestogen preparations in perimenopausal women has been shown to increase breast density. This results in breast screening being less sensitive and specific [12]. As a result, the detection of breast cancer can be delayed.

Recent large prospective studies have reported increased breast cancer rates, particularly for preparations containing a combination of oestrogen and progestogen. The Million Women Cohort Study [13] enrolled 1084110 women aged 50-64 who attended for breast screening between 1996 and 2001. Half of these women had taken HRT at some time. There was an average follow up of 2.6 years for incidence and 4.1 years for mortality. Current users of HRT were found to be 1.66 times more likely to develop breast cancer

than those who had never used HRT. The risk was doubled with oestrogen and progestogen containing preparations [13].

It has also been shown that the longer HRT is used, the greater the risk. It remains unclear whether breast cancer mortality is higher in women who have used HRT than in those who have not. The Million Women study reported a 1.22 relative increase in risk of death from breast cancer in 'current users' compared with 'never users' [13]. However, longer follow up is required to confirm the findings.

The issue of HRT use in breast cancer survivors also remains unclear. Initial small scale studies were reassuring about its safety but the HABITS study in Sweden was terminated early because of a significant increase in breast cancer events in women randomised to receive HRT.

Safer alternatives to HRT such as tibolone have been suggested. Tibolone is a synthetic steroid which has been shown to have weak oestrogenic, androgenic and progestogenic activity. It has been shown to relieve menopausal symptoms to a similar degree as combined HRT but does not appear to have the same adverse effects on the endometrium and has beneficial effects in protecting against osteoporosis. It does not appear to increase breast density to the same extent as HRT although its effects on the breast have not been fully investigated. However, the Million Women Study showed an increased risk of breast cancer of 1.44 with tibolone although there was no increased risk of endometrial cancer.

The full effect of the widespread use of HRT over the past few decades remains to be seen. It also remains a challenge to treat menopausal symptoms while limiting the risks associated with increased exposure to hormonal therapy.

1.2.4 Other risk factors

Other risk factors include postmenopausal obesity, proliferative conditions such as atypical ductal hyperplasia and previous breast irradiation[9]. Dietary factors may be important in the substantial difference in incidence between different geographical areas. It has been suggested that a diet rich in saturated fat may increase risk especially if consumed when young. Olive oil may have a protective effect [14]. Smoking has not been shown to increase risk but alcohol consumption of greater than three units per day does appear to increase risk [15].

1.3 The role of oestrogen in the normal breast

Histologically the breast is a mammary gland which comprises a rudimentary branching duct system within a fat pad. After puberty there are cyclical increases in the ductal branching to fill the fat pad. During pregnancy the branches develop further and end buds develop which produce milk. After weaning, apoptosis causes mammary gland regression to a pre pregnancy state. The ductal structure consists of a basement membrane, myoepithelial cells and a continuous layer of epithelial cells which produce milk and secrete it into the lumen. There are fibroblasts surrounding the cell layers.

The breast requires oestrogen in order to develop normally at puberty. The reduction in circulating oestrogen at menopause results in breast involution and replacement of epithelial tissue with fat. Between these times the breast undergoes cyclical growth and involution in response to the menstrual cycle and to pregnancy and lactation.

1.3.1 The oestrogen receptor

The effects of oestrogen on the breast are mediated by the oestrogen receptor (ER). These receptors are present in the epithelial cells of the terminal duct lobular unit which is where the majority of breast cancers originate. ERs are present on the epithelial cells of normal breasts. However, in the majority of breast cancers ERs are found at levels greater than in normal breast tissue. In the resting breast between 15 and 25% of epithelial cells are ER positive. The level of ER expression varies throughout the menstrual cycle with lowest levels during the luteal phase (corresponding to highest levels of circulating oestrogen). This corresponds with the maximal proliferation of epithelial cells in the breast. What triggers ER positive cells to proliferate in response to oestrogen remains unclear.

The majority of oestrogen in premenopausal women is produced by the ovaries. In postmenopausal women, the ovaries cease to produce oestrogen. The majority of oestrogen in plasma is converted from androgens (eg androstenedione and dehydroepiandrosterone secreted from the adrenal glands) by the enzyme aromatase in peripheral tissues such as fat, liver and muscle. In postmenopausal breast cancer, the intra-tumoural concentrations of 17B-oestradiol (oestrogen's main circulating form) are more than 20 times higher than in plasma [16]. This is likely to result from breast tumours having either greater levels of uptake or high levels of synthesis and so high levels of aromatase, the enzyme which synthesizes oestrogens from adrenal steroids.

There is considerable evidence that the risk of breast cancer increases with prolonged exposure to oestrogens. The exact mechanism for this remains unclear. One possibility is that the proliferative effect of oestrogens on the breast promotes malignant cell proliferation and breast cancer progression. Approximately two thirds of breast cancers are ER positive, compared with 15-25% of normal breast epithelial cells. Additionally, ER expression is higher in benign breast epithelial tissue in women with breast cancer than in women who do not have breast cancer. This suggests that ER expression may have a role in early events that lead to the development of breast cancer.

The highest ER values are found in postmenopausal women with well differentiated tumours. The expression of ER is inversely correlated with proliferation rates in breast cancers. This is in keeping with the finding that ER and Progesterone receptor (PgR) are more likely to be expressed by well differentiated tumours which have lower degrees of proliferation and a better prognosis. The value of ER alone as a prognostic factor is weak and gradually decreases with time.

Interestingly, it has been suggested that ER positive cells may promote proliferation of surrounding ER negative cells rather than proliferating themselves [16].

1.4 Management of breast cancer

1.4.1 Diagnosis

In the UK, women with a breast lump usually first present to a general practitioner who refers them to a breast clinic which is usually run by a general surgeon with a special interest in breast surgery. Women who are treated by specialists, who see large numbers of patients with breast cancer and who work as part of a multidisciplinary team, have better clinical outcomes [17]. Currently, patients who present to a breast clinic with a breast lump have a detailed history taken and are investigated using triple assessment. This involves clinical examination, imaging and cytohistological assessment.

Imaging routinely involves mammography for all women over 35 years of age and often an ultrasound (US) scan of any palpable lump for women under 35. Pathological assessment includes fine needle aspiration (FNA) of any breast lump and results are usually available within one hour. This can be done freehand on any palpable lump or under ultrasound or mammographic visualisation if the lump is not palpable. Increasing use is being made of 14 gauge core biopsy either in addition to or as replacement for FNA. This gives additional information preoperatively, including the presence of invasion and oestrogen receptor (ER) status.

After a woman has been diagnosed with breast cancer she will typically have routine staging investigations performed. This involves blood tests (full blood count, urea & electrolytes, and liver function tests) and chest x-ray (CXR) for women with early stage disease. It may also include liver ultrasound and bone scan for women with more advanced disease.

1.4.2 Use of core biopsy

Fine needle aspiration cytology has been the first line pathological diagnostic test in assessing breast lumps for many years. It has high sensitivity and specificity [18]. Increasingly, core biopsy is being performed in addition to FNA or in preference to it. Core biopsy has been shown to increase the rate of diagnosis of both invasive and pre invasive breast cancers. Tissue from core biopsies is extremely useful in treating advanced breast cancer as it can be used to assess prognostic markers such as ER which may influence the choice of primary endocrine or primary chemotherapy treatment. It is also useful for research purposes as it can be used as a baseline for assessments prior to starting any systemic therapy. Cores can also be obtained during treatment at different time intervals to assess pathological response and compared with final tumour excision specimens. Core biopsy specimens do have some potential drawbacks because of their relatively small size. Known tumour heterogeneity may lead to differences in results that are due to sampling technique rather than as a result of treatment. However there is generally good concordance between core biopsies and tumour sections in relation to several pathological variables such as oestrogen receptor [19].

1.4.3 Treatment of breast cancer

Staging

Breast cancer is currently staged using the TNM (primary stage, regional nodes, metastasis) classification (Table 1):

Tis	In situ disease only
T1	<2cm
T2	>2 – 5cm
T3	>5cm
T4a	Chest wall involved
T4b	Skin involved
T4c	Both skin and chest wall involved
T4d	Inflammatory cancer
N0	no regional node metastasis
N1	mobile ipsilateral nodes
N2	fixed ipsilateral nodes
N3	internal mammary nodes involved
M0	no evidence of metastasis
M1	distant metastasis

Table 1: Classification of breast cancer using TNM staging

The treatment of breast cancer varies according to the stage at which it presents. If untreated, approximately 20% of patients will be alive five years after diagnosis and 5% will be alive after 10 years. Patients with early stage breast cancer who are treated appropriately now have over an 80% probability of being alive five years later.

1.4.3.1 Early breast cancer

Women with early breast cancer T1-3, N0-1, M0 are those considered to have operable disease. Their primary treatment therefore consists of surgery. The aim is to cure by excising loco-regional disease and treat potential micro metastases. In addition to surgery, adjuvant radiotherapy, chemotherapy and/or endocrine therapy can be used depending on individual circumstances.

1.4.3.2 Locally advanced breast cancer

Some patients with locally advanced (usually inoperable) breast cancer but no evidence of metastasis can be treated with neoadjuvant therapy to down stage their tumour and allow surgical excision. In this group, after primary treatment they can be treated in the same way as patients with early breast cancer and given post-operative adjuvant treatment with the aim of cure. If they have had successful neoadjuvant endocrine treatment, they can continue on the same drug therapy post operatively with the advantage of knowing that their tumour is sensitive to the treatment.

1.4.3.3 Advanced breast cancer

Women with advanced or inoperable breast cancer are treated differently. The main aims of treatment are to control local disease and maintain quality of life. With the advent of more effective endocrine agents and chemotherapy it is now also possible to prolong survival. In this group, it is important to remember the impact of any intervention on quality of life. The main goal of treatment remains palliation although some patients with associated comorbidity may not die as a direct result of their breast cancer. These women can be treated with primary (neoadjuvant) endocrine therapy or chemotherapy. Another option in this group is to palliate local or metastatic disease using radiotherapy.

1.4.4 Surgical treatment of the breast and axilla

Surgical treatment of breast cancer consists of excision of the primary breast cancer and draining regional lymph nodes from the corresponding axilla.

Over the past decade there has been a move away from mastectomy towards breast conserving surgery. This consists of excision of the tumour with a surrounding 1cm macroscopic margin of normal tissue.

Several trials have shown similar rates of survival between patients treated with breast conserving surgery and mastectomy [20]. However, breast conserving surgery has a higher rate of local recurrence than mastectomy unless it is followed by postoperative radiotherapy [21]. Therefore, for women with a single primary tumour less than 4cm in size that is not locally advanced and does not have extensive nodal involvement or evidence of metastasis, the treatment of choice is wide local excision followed by radiotherapy. Breast conservation is not possible or appropriate for all tumours eg. multifocal breast cancers or those surrounded by large areas of ductal carcinoma in situ (DCIS). Mastectomy may also give a better result for women with larger tumours in small breasts.

The role of axillary surgery in breast cancer is both to stage the axilla and to treat any axillary disease that may be present. There have been some changes in management of

axillary surgery over the past few years. Previously, most patients with breast cancer were routinely treated with a level II or III axillary clearance. Since half of symptomatic breast cancers and 80-90% of screen detected cancers have no nodes involved, a significant proportion of patients were having unnecessary surgery sometimes resulting in significant morbidity in terms of lymphoedema and nerve damage [22].

Over the past few years, there has been a move towards axillary sampling (taking 3 or 4 nodes from level I) and sentinel node biopsy rather than clearing the axilla for smaller tumours. Since size of primary tumour is directly related to likelihood of presence of axillary metastasis, many centres treat patients with small tumours (<2cm) who are clinically node negative with axillary sampling. Sentinel node biopsy of the axilla has now taken over as the method of determining which patients require either an axillary clearance or post operative radiotherapy to the axilla. This is being introduced gradually in all patients undergoing breast conserving surgery and in many patients undergoing mastectomy who are clinically node negative.

1.4.5 Factors influencing breast cancer prognosis

Breast cancer differs from other epithelial tumours in that it can have an extended clinical course over up to 30 years. This means that decisions regarding adjuvant treatment need to be guided by prognosis. The most important factors in influencing prognosis are tumour size at presentation, degree of involvement of axillary lymph nodes and tumour grade. The most widely used tool in calculating prognosis and aiding decision-making in adjuvant therapy in the UK is the Nottingham prognostic index (NPI).

The NPI was constructed in 1982 for patients with primary, operable breast cancer. The index was based on a retrospective analysis of nine risk factors in 387 patients. Only three of the factors (tumour size, stage of disease, and tumour grade) remained significant on multivariate analysis. The NPI uses lymph-node stage, tumour size and pathological grade to calculate a score using the formula (0.2 x size of invasive cancer in cm) + lymph node stage + grade. Lymph node stage is assigned as follows,

1 if no nodes are involved

2 if one to three nodes are involved

3 if four or more nodes are involved.

The NPI has been subsequently validated in several prospective studies.

The index can be used to define a number of groups of patients clustered according to their prognosis. Originally the NPI was used to divide women into good, intermediate or poor prognostic groups. The good prognosis group comprises approximately 30% of patients and has a survival close to that of age matched controls (83% 15-year survival). This group benefits little from aggressive adjuvant therapy. In contrast, the poor prognostic group (17% of patients) has only a 13% 15-year survival and therefore may well derive benefit from aggressive systemic therapy. Recently the NPI has been refined and further divided into 6 groups based on 10 year survival (table 2).

Prognostic Group	NPI value	10-year survival (%) 1990-1996	10-year survival (%) 1980-1986
Excellent	≤ 2.4	96	94
Good	2.41 - 3.4	93	83
Moderate I	3.41 - 4.4	82	70
Moderate II	4.41 - 5.4	75	51
Poor	5.41- 6.4	53	}19
Very poor	≥ 6.41	39	}19

Table 2: Nottingham Prognostic Index [23;24]

There has been a dramatic improvement in survival over the last decade. The 10-year survival data shown in table 2 is from patients with primary operable breast cancer treated between 1990 and 1996 (Nottingham Tenovus Primary Breast Cancer Series) and 1980-86 [25].

1.4.6 Adjuvant treatment of breast cancer

In the 1970s, it became obvious that locoregional treatment alone for breast cancer was insufficient to control disease. Patients were dying from metastatic disease, probably from occult micrometastases that were already present at the time of diagnosis and subsequent surgery. As a result, the use of adjuvant systemic treatment has become routine. This consists of either chemotherapy or endocrine therapy or a combination of both. Interest in the importance of ER status in breast cancer increased as it became apparent from trials that patients who benefited most from hormone therapy were patients with ER positive cancers. However, those who benefited most from chemotherapy were young women with ER negative tumours [26]. Chemotherapy does produce some improvement in survival in postmenopausal women although the improvement is not as great as in those women who are still menstruating.

Until recently almost all women with ER positive tumours were treated with five years of adjuvant tamoxifen. As more data has become available from the adjuvant studies comparing tamoxifen with aromatase inhibitors, increasing numbers of women especially those with higher risk cancers are being treated with an aromatase inhibitor. This varies between starting adjuvant treatment with an AI, switching from tamoxifen to an AI after two or three years or having extended adjuvant therapy with an AI after completing five years of tamoxifen. Treatment is tailored according to an individual patients risk profile.

Radiotherapy is used routinely for all women who have breast conserving surgery and for selected women identified to be at relatively higher risk of local recurrence after mastectomy.

1.4.7 Neoadjuvant treatment of breast cancer

Traditionally, breast cancer treatment has consisted of surgery followed by radiotherapy and then adjuvant systemic therapy. Fewer women are now treated surgically by mastectomy and more by breast conserving surgery. To extend the use of breast conserving surgery, there has been increasing use of both primary (neoadjuvant) chemotherapy and endocrine therapy to reduce tumour size prior to surgery [27-35].

Until recently, neoadjuvant protocols in breast cancer have most frequently utilised chemotherapy to downstage the size of the primary tumour and allow more breast conserving surgery to be performed. Both disease-free survival and overall survival have been reported to be similar in patients treated with neoadjuvant chemotherapy preoperatively and in patients treated with systemic therapy after surgery [28-30;32] although significantly more patients can be treated without mastectomy following preoperative chemotherapy. It is of interest that complete response rates with chemotherapy are lower in patients whose tumours are ER positive compared to patients who are ER negative [36;37].

In the past, endocrine agents were used as sole therapy without surgery in elderly patients whose tumours fortunately tend to be ER positive. Such agents are much better tolerated than chemotherapy. Later tamoxifen was used to downstage the surgical procedure which could be performed. This is important in elderly patients who tend to

have other significant comorbidity and have significant risk factors for surgical complications.

Tamoxifen was the first widely used neoadjuvant endocrine treatment and was successful in elderly patients at reducing the size of tumours[38]. This allowed inoperable tumours to become operable and tumours which would have required mastectomy to be removed by wide local excision [33-35;39]. However studies which compared tamoxifen treatment alone with tamoxifen followed by surgery showed that, although most tumours responded initially, local disease control was poor with endocrine therapy alone [31;33-35]. Further, survival from breast cancer was poorer in the tamoxifen alone group [40]. More recent studies have shown that letrozole is a more effective agent than tamoxifen in this setting in postmenopausal women [41].

1.5 Breast cancer in the elderly patient

30% of breast cancer occurs in women aged over 70 and 48% in those aged over 65 [42;43]. These figures are likely to increase in line with the growth in the elderly population. The elderly are excluded from many clinical trials despite the fact that studies have shown they are willing to participate [44]. Elderly patients differ in a number of ways which might affect the outcome of treatment. They have a shorter life expectancy, increased co-morbidity and different psychological and functional profiles [45-47]. There is also the possibility that breast cancer in the elderly behaves in a biologically different way, manifesting itself as a less aggressive disease. These factors mean that it is increasingly important to include the elderly population with breast cancer in clinical trials and to investigate the disease in this population.

Survival data in the elderly are notoriously difficult to interpret because of comorbidity. Many patients with breast cancer die from unrelated conditions. Breast cancer causes 73% of deaths in affected patients aged between 50 and 54 but only 29% in those aged over 85 [48]. However, life expectancy is often longer than realized at the extremes of age. At age 70, the average life expectancy is 15 years but at age 80 it is 8 years [45]. It is therefore particularly important to treat breast cancer appropriately in the elderly population.

Cosmetic treatment of breast cancer has often been considered an area more relevant to younger women than the elderly. In terms of making the choice between breast conserving surgery and mastectomy, younger women are more likely to be offered breast conservation [49]. However, the only study to have examined patient choice in women over 70 reported that the majority of women with early breast cancer would prefer BCS. They were fully prepared to go through a full course of radiotherapy in order to optimize the cosmetic result and local disease control [50]. Only one of 31 patients considered that radiotherapy would be 'too much trouble'. The elderly are also less likely to want to be involved in making treatment decisions and are happier to allow doctors to advise them. They are also less likely than younger women to ask questions or to seek a second opinion [51].

1.5.1 Biology of breast cancer in the elderly

Breast cancer in the elderly has traditionally been considered to be less biologically aggressive than in younger women. There is evidence of greater sensitivity to oestrogen with a higher proportion of tumours being ER positive. There are also small but significant differences in other markers of aggressive disease eg grade and HER2 expression [51]. There are a higher number of more differentiated tumours such as mucinous, lobular and papillary breast cancer (Table 3). [48;52-54].

Ref	Biological factor	Age group (years)	%	Age group (years)	%	Significance (p value)
37	Poorly differentiated	<71	39	>71	24	<0.001
28	Lobular	55-64	8	>65	9.1	<0.001
28	Mucinous	55-64	1	>65	3	<0.001
40	Papillary	35-69	0.2-1	70-85	1.6-2	NS
37	ER positive	<71	59	>71	77	<0.001
37	PgR positive	<71	51	>71	63	NS

Table 3: Incidence of different histological types of breast cancer according to age

1.5.2 Response to tamoxifen in elderly patients

In the early 1980s several studies reported the use of tamoxifen as primary endocrine therapy for breast cancer in the over 70s age group [55-59]. The initial results were promising, with significant numbers of patients achieving response or stasis in their tumour after a year of treatment. After ER status was taken into account, a good response to treatment could be expected in 79 to 90% of patients whose tumours were moderately or strongly ER positive [55;56;60;61]. This was an attractive option for elderly patients and for surgeons and many patients were treated in this way. However, with time it became apparent that the major drawback of treatment was the short duration of response and disease control (mean response 18-24 months) [55;60]. This meant patients required to change to second line treatment or face surgery or radiotherapy when they were two years older than when first diagnosed.

Based on long term results (after 12 years of follow up), 38% of women develop progressive disease after surgery, compared with 81% of women treated with primary tamoxifen. There is also a significant reduction in survival in the tamoxifen-only group [62]. However, there was a small group of long term responders (19% in one study) [63] who had good local control 12 years after primary tamoxifen treatment. They had been spared surgery. If it was possible to identify this subgroup there may still be a place for primary endocrine therapy alone. Out with this small subgroup of patients, primary endocrine therapy is still best combined with surgery to obtain optimal disease control.

1.5.3 Surgical treatment in the elderly

Mastectomy carries small but significant morbidity and mortality rates. Morbidity is approx 19% in those over 65 and does not increase significantly with age [51]. The majority of complications are wound seromas, haematomas and infections which can be resolved relatively easily. Mortality is 1% in patients over 65 [64;65]. There are lower rates of morbidity and mortality with breast conserving surgery than with mastectomy. Mortality in patients over 70, having had breast conserving surgery, has been reported to be 0.3%. [51]. Elderly patients are more likely to be offered BCS than mastectomy, probably because it is easier to perform BCS under local anaesthesia [43;48;66;67]. Axillary surgery is more likely to be omitted in the elderly as is adjuvant radiotherapy [51]. Several studies have reported local recurrence rates varying between 3 and 47% after wide local excision alone without radiotherapy [33;35;68-71]. The mean time from operation to local recurrence is short therefore necessitating further treatment.

1.5.4 Axillary surgery in the elderly

Only 10% of elderly patients present with palpable axillary disease but approximately a third of all axillary clearance specimens are node positive [51]. A review of studies in the elderly where no axillary surgery was performed (1873 patients in total) showed an axillary recurrence rate of 12.6% after follow up of 3-10 years. However, in only 0.3% of cases the disease was not able to be controlled by operation or radiotherapy [51].

The risks of axillary clearance are no higher in the elderly population although the morbidity is higher than when breast surgery alone is performed. This is true of all age groups [72;73]. Younger patients have a worse outcome following axillary surgery in terms of restriction of daily activities than older people, presumably because they were generally more active. The use of axillary sampling or sentinel node biopsy allows elderly patients the advantages of accurate staging without the increased morbidity of clearance.

There is a concern that women who fail to respond to primary endocrine therapy have had a delay in effective loco-regional treatment. This is obviously a particularly important factor for elderly patients as one or two years can make a significant difference to whether or not they may be considered fit for surgery.

1.6 Endocrine treatment of breast cancer

The growth of many breast cancers is dependent upon hormones, most notably oestrogen, and endocrine deprivation is therefore a major treatment modality. It is well established that oophrectomy or ovarian ablation can result in tumour regression. Over a century ago, it was first appreciated that approximately one third of patients with advanced disease responded following oophrectomy. Since then various forms of surgical endocrine manipulation have been used in the treatment of breast cancer including hypophysectomy and adrenalectomy. However, these procedures had significant associated morbidity requiring replacement corticosteroid therapy administration, and have therefore been replaced by drugs which result in hormone deprivation.

Lutenising hormone releasing hormone (LHRH) analogues (eg. goserelin) can now be used to cause a 'medical oophrectomy' in premenopausal women thus avoiding the need for surgery. However, this is often achieved as a useful "side effect" of cytotoxic chemotherapy.

Tamoxifen has been the endocrine agent of choice since the 1970s. However, over recent years there has been increasing use of aromatase inhibitors to treat breast cancer. It is likely that they will replace tamoxifen as the agents of first choice in postmenopausal women over the next five years.

1.7 Tamoxifen use in breast cancer

Tamoxifen was first used in patients with advanced disease. It produced a clinical response in approximately one third of women treated. Initially it was given to all patients, regardless of ER status but it gradually became apparent that it was only effective in those who were ER positive. It is currently used as an adjuvant treatment for both pre and postmenopausal women with breast cancer.

Tamoxifen is a selective oestrogen receptor modulator and has now been established for nearly 30 years as standard first line hormonal therapy for ER positive tumours. It is routinely given both as adjuvant therapy for five years postoperatively in low risk women and used alone or in combination with other treatments in advanced breast cancer [74].

Tamoxifen antagonises ER function by binding competitively to the receptor but it also has partial agonist properties. Its activity in breast cancer appears to be due to its anti-oestrogenic effects. Extensive clinical studies have confirmed tamoxifen's efficacy in both early and advanced breast cancer [75].

1.7.1 Use of tamoxifen in the adjuvant setting

As the concept of early micrometastasis in breast cancer became established a need was identified to treat women with systemic adjuvant therapy after primary locoregional therapies. The aim of systemic adjuvant therapy was to eradicate or control these micrometastases without giving the patient further significant adverse events. Tamoxifen has been shown to decrease both recurrence rates and mortality when compared with no adjuvant treatment [74-76]. In addition, it reduces the rate of contralateral breast cancer development when compared with placebos. As predicted, it is most effective in ER positive tumours.

There has been much discussion about the optimal duration of adjuvant tamoxifen treatment [74]. However, using tamoxifen for more than five years has not been shown to provide any additional benefit. In fact, outcomes may be worse because tamoxifen is known to have adverse side effects eg two-three times increased risk of endometrial cancer and increased thromboembolic events. Therefore, five years is the current standard adjuvant period for women with low risk breast cancer being treated with tamoxifen alone. As aromatase inhibitors are being increasingly used in the adjuvant setting, tamoxifen alone is currently reserved only for women with a very low risk of recurrence.

1.7.2 Use of tamoxifen in the neoadjuvant setting

Tamoxifen was first used for the treatment of advanced disease. An early study of 1200 patients in this setting showed a 32% response rate to tamoxifen [77]. This rose to 50% when only ER positive patients were considered [78]. Additionally, there was increased survival in the group of patients that responded. Tamoxifen has also been studied widely in the neoadjuvant setting. It is relatively well tolerated by most patients and does not have the significant associated morbidity of neoadjuvant chemotherapy. However, as previously described, when used alone it has been shown to have low rates of long term local disease control and is best used in combination with surgery. There has been growing interest in the past few years of the use of aromatase inhibitors in this setting. Following the publication of the PO24 study which showed letrozole to be more effective in the neoadjuvant setting than tamoxifen, letrozole has become the agent of first choice in this setting [41].

1.8 Use of aromatase inhibitors in breast cancer

Aromatase inhibitors (AIs) work by blocking oestrogen production via inhibition of the aromatase enzyme [79]. Aromatase is a cytochrome P-450 dependent enzyme that converts androgen substrates to oestrogens. In postmenopausal women, the major sites of aromatase are in peripheral tissues including adipose tissue, muscle, skin and both benign and malignant breast tissue. Third generation AIs are able to reduce circulating plasma oestrogen levels to below detectable limits in post menopausal women [80;81]. Over the past decade, there has been increasing use of aromatase inhibitors in the treatment of ER positive breast cancer in postmenopausal women.

Aminoglutethimide was the first AI used clinically. It had similar efficacy to tamoxifen but adverse effects limited its clinical use. Second generation inhibitors were developed which were less toxic but it was not until third generation AIs appeared that their clinical use expanded. These third generation AIs are extremely potent inhibitors of the aromatase enzyme (inhibiting oestrogen synthesis by 97-99% compared with 85-90% for second generation compounds[82]). There are also data comparing these newer agents and suggesting that letrozole is a more complete inhibitor of oestrogen synthesis than anastrozole [81;83]AIs in current use result in near maximal oestrogen suppression [84;85], but unlike the earlier aromatase inhibitor aminoglutethimide, they are selective for the aromatase enzyme and do not interfere with production of other steroid hormones such

as adrenal corticosteroids [79]. Consequently, they do not need to be given with corticosteroid replacement, as was the case with aminoglutethimide.

There are two different groups of AIs in current use. The first group includes the type I inhibitors or inactivators such as exemestane. These are generally androgen analogues which are steroidal and interact directly at the substrate molecule-binding site. They bind irreversibly hence the term "inactivator". The second group, known as type II aromatase inhibitors, are non-steroidal and include anastrozole and letrozole (Figure 3). They inhibit aromatase by binding reversibly to the adjacent heme site of the enzyme. This blocks the access of androgens to the substrate molecule binding site, so preventing oestrogen production. The different mechanisms of action of inactivators and inhibitors may have implications for the ability of breast cancers to develop resistance to these two types of aromatase inhibitor. There is some clinical data to suggest that tumours, which are no longer responding to type II inhibitors such as letrozole or anastrozole, can still respond to the type I inhibitor exemestane [86].

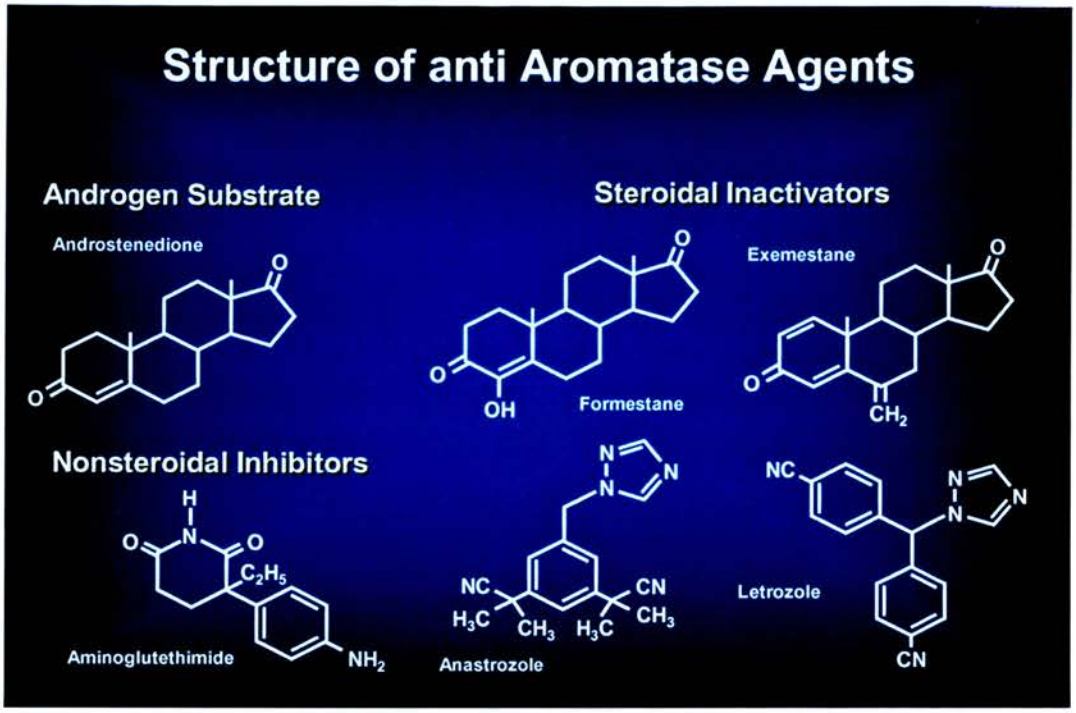

Figure 3: Structure of Aromatase inhibitors

Since the mid 1990s, these orally active, potent, selective third-generation aromatase inhibitors have been used in the treatment of metastatic breast cancer in postmenopausal women with oestrogen receptor positive tumours. Randomised clinical trials have established their superiority over megestrol acetate in the second line setting which was formerly the drug most commonly used after tamoxifen failure [87-89]. More recent studies suggest that aromatase inhibitors are superior to tamoxifen when given as first line therapy in advanced breast cancer [90-92].

1.8.1 Aromatase inhibitors in the adjuvant setting

Tamoxifen, given as adjuvant hormonal therapy for breast cancer, dramatically reduces the risk of relapse after surgery in early breast cancer. Five years of treatment with tamoxifen reduces the risk of recurrence by 47% and the risk of death by 26% in ER positive patients [93]. AIs have the potential to be even more effective in this setting and recent reports of large trials indicate a better outcome for women given AIs compared with tamoxifen in the adjuvant setting [94;95].

The ATAC (Arimidex, Tamoxifen Alone or in Combination) trial was the first large randomised controlled trial of an adjuvant AI for which results have been published. It compared the aromatase inhibitor anastrozole with tamoxifen. Between July 1996 and March 2000, 9366 postmenopausal women with invasive breast cancer from 381 centres in 21 countries were recruited. After completing primary therapy (surgery +/- radiotherapy +/- chemotherapy) women were randomised to receive anastrozole plus placebo, tamoxifen plus placebo or anastrozole and tamoxifen together for five years as adjuvant therapy. Patients in each of the groups had similar demographics and tumour characteristics and had similar primary treatment of their cancer. In each group, approximately 83% of tumours were oestrogen receptor positive, 7% were oestrogen receptor negative and 10% were unknown. Updated results were published in 2004 after a median follow up of 68 months [96].

Anastrozole was superior to tamoxifen in terms of disease free survival in both the overall study population (hazard ratio 0.87 [95% CI 0.78-0.97], p=0.01) and the oestrogen receptor positive subgroup. Anastrozole was also superior to tamoxifen in terms of time to recurrence (0.79[0.70-0.90], p=0.0005) and time to distant recurrence (0.86 [0.74-0.99], p=0.04). There were also significantly fewer new contralateral primary tumours in the anastrozole treated group (42% reduction [95% CI 12-62] p=0.01 for all patients; 53% for hormone receptor positive patients [95% CI 25-71 p=0.001). Interestingly the combined arm did not show any significant improvement over tamoxifen alone.

The breast international group (BIG) 1-98 study compared not only letrozole alone with tamoxifen as initial adjuvant endocrine therapy but also sequential treatment with the two agents in either order. The study involved 8010 postmenopausal women with hormone receptor positive breast cancer [93]. Only data from the direct comparison between letrozole and tamoxifen is currently available. After a median follow up of 25.8 months, 351 events had occurred in the letrozole group compared with 428 in the tamoxifen group, with five year survival estimates of 84% and 81.4% respectively. Letrozole significantly reduced the risk of an event ending a period of disease free survival when compared with tamoxifen (hazard ratio 0.81; 95% CI 0.7-0.93; p=0.003). This was particularly the case when looking at distant recurrence (hazard ratio 0.73; p = 0.001) which is what is likely to impact most on survival.

1.8.2 Extended adjuvant therapy

Because of the lack of cross resistance between tamoxifen and AIs and their different mechanisms of action there has been interest in the extended use of adjuvant endocrine therapy with AIs after completion of five years of adjuvant tamoxifen.

The double-blind, placebo-controlled MA17 trial was designed to evaluate whether postmenopausal women with hormone receptor-positive early breast cancer, who had completed five years of adjuvant treatment with tamoxifen could benefit from receiving an additional five years of treatment with letrozole [97]. 5187 patients were enrolled with a median follow-up of 2.4 years. At the first analysis, there was a significant difference in disease free survival (DFS) (P <0.001), with 75 recurrences or new primary contralateral breast cancers in the letrozole group compared with 132 in the placebo group. The estimated four year DFS rates were significantly higher for letrozole (93% and 87% for letrozole and placebo, respectively; $P \leq 0.001$). On the basis of these results, the data and safety monitoring committee recommended that the trial should be terminated early and the results of this interim analysis be published. Updated results were presented at ASCO 2004, indicating that letrozole was associated with a 43% reduction in risk, producing an absolute improvement in three year DFS of 3% and 7% in patients with node-negative and node-positive disease respectively.

As a result of the early discontinuation of the trial, the study did not achieve its main aim of determining DFS and overall survival in women switching from tamoxifen to letrozole or placebo for five years. Thus the optimal duration of letrozole therapy in this context remains undefined. Several studies are ongoing in an attempt to determine the optimum duration of extended adjuvant treatment. Some recent preliminary data suggests that at least 10 years adjuvant treatment should be used for node positive patients and that even patients who have had a gap since stopping tamoxifen could benefit from starting on letrozole.

1.8.3 Sequencing adjuvant therapy

A benefit has been seen in switching from tamoxifen to an AI after two to three years for both anastrozole and exemestane. The intergroup exemestane study (IES) randomised patients who had been on tamoxifen for two or three years. They switched to exemestane or continued with tamoxifen for up to five years [95]. Switching to exemestane improved relapse free survival and metastasis free survival. Combined analysis of the ARNO and ABCSG trials, which compared anastrozole with tamoxifen with a similar trial design to IES, showed similar beneficial outcomes in event free survival after switching to anastrozole [98]. The rationale behind such a treatment strategy is that initial treatment with tamoxifen may 'sensitise' micrometastatic disease to aromatase inhibition. There are several ongoing trials which hope to address the optimal time to switch and the optimal sequence of drugs to choose.

1.8.4 Summary of optimal adjuvant AI use

In summary, in two Phase III trials comparing an AI with tamoxifen for the adjuvant treatment of breast cancer in postmenopausal women, disease-free survival was significantly improved with anastrozole and letrozole compared with tamoxifen as initial adjuvant treatment ($P = 0.01$ and $P = 0.003$, respectively) [93;94]. A switch to either anastrozole or exemestane after two to three years of adjuvant tamoxifen therapy was more effective in reducing the risk of recurrence than continuing with tamoxifen therapy ($P = 0.006$, $P < 0.002$, and $P < 0.001$, respectively) [95;98]. In another Phase III trial, letrozole was found to improve disease-free survival in the extended adjuvant setting ($P < 0.001$) [97] and was the only AI consistently more effective than tamoxifen in the neoadjuvant setting [41].

The use of adjuvant tamoxifen and AIs is complex at present. Evidence from recent studies and numerous ongoing studies which aim to clarify the optimal choice of drug in each setting have not been entirely conclusive and further results are awaited before a clear treatment strategy emerges. Their use has to date been based on available evidence from trials and each individual drug's licensed indications. At present, women considered to be at low risk of recurrence are offered five years of adjuvant tamoxifen. Women with contraindications to taking tamoxifen and those considered to be at high risk of early relapse on tamoxifen alone are offered an upfront AI.

A retrospective analysis of the Edinburgh Breast Unit database looking at risk factors for relapse in women who took five years of adjuvant tamoxifen between 1981 and 1998 was performed. 670 women were identified who had breast conserving surgery, of whom 121 had relapsed to date [99]. Risk factors for relapse in the first 2.5 years were ER poor tumours, Grade 3 tumours and 4+ positive nodes. These are the patients who the authors felt should be considered for an up front AI. The only individuals who did not have a significant relapse rate within the first five years were those with Grade 1 tumours who could be considered for tamoxifen alone. They concluded that all other patients should be considered for switching to an AI after two to three years of treatment with tamoxifen as there were no particular features identified which predicted for mid- to late relapse. Beyond five years only tumour grade and number of lymph nodes involved were predictors for recurrence.

Until a clearer picture emerges about the optimal place for aromatase inhibitors and tamoxifen in the adjuvant setting, it is likely that different units will have different policies about who to treat with which agents and in which sequence. This will probably be tailored according to the patient's individual risk profile for recurrence. Cost of treatment and side effects will also need to be taken into account and more data is constantly emerging regarding these factors.

In a patient-preference study, those receiving letrozole reported fewer adverse events than those receiving anastrozole (43% vs 65%; $P < 0.003$), and more patients preferred letrozole to anastrozole (68% vs 32%; $P < 0.01$) [100].

1.8.5 Side effects of Aromatase inhibitors

Previous studies, comparing aromatase inhibitors with tamoxifen, have shown a similar or an improved side effect profile for the AIs. The ATAC study showed anastrozole treated patients had a lower incidence of thromboembolic events, vaginal bleeding and endometrial carcinoma than those treated with tamoxifen [94;101]. However, since aromatase inhibitors lower oestrogen levels so potently, women in the ATAC study had problems with a higher rate of bone fractures since oestrogens are important for bone metabolism. The AIs may turn out to have an adverse effect on lipid metabolism as well and there have been concerns about potentially increased cardiovascular morbidity and mortality. However, findings from studies to date have not been consistent with regard to hypercholesterolaemia and the reported differences in cardiac events between tamoxifen and AIs are likely to be due to the protective effect that tamoxifen has on lipid profile rather than an adverse effect from the AIs.

There is concern about the osteoporotic effects of AIs as the concentration of oestradiol becomes virtually undetectable in patients on AIs. It is much lower than in normal postmenopausal women and that normally required to maintain good bone health. However, when comparing the increased fracture rates seen in the adjuvant AI trials it is evident that the fracture rates with AIs are only marginally worse than those seen with placebo and that rates seen with tamoxifen treated patients are similar to those seen in postmenopausal women on HRT. Therefore, it is likely that the bone changes seen in

the AI trials are probably due to the absence of, or switching from, tamoxifen rather than simply due to the AI. It is also likely that bisphosphonates can be used to treat effectively any significant adverse effect on bone. For this reason, patients embarking on treatment with an AI should have a baseline DEXA scan to assess their bone mineral density and to guide any potential requirement for treatment with bisphosphonates.

AIs may also have a role in breast cancer prevention [102]. In settings in which apparently well women are taking AIs, side effects, morbidity and tolerability become as important as the anti-tumour properties of the drugs.

In advanced breast cancer, side effects of aromatase inhibitors have resulted in treatment being stopped in less than 4% of women[103].

1.8.6 The use of aromatase inhibitors in the neoadjuvant setting

With the recent expansion of the use of aromatase inhibitors in postmenopausal breast cancer patients the scope for using them in the neoadjuvant setting has increased.

Initial small scale pilot studies in Edinburgh compared the use of all three currently available aromatase inhibitors with tamoxifen in the neoadjuvant setting [101;104;105]. These studies were non-randomised or randomised to different doses of an aromatase inhibitor. Patients selected were postmenopausal and had large operable or locally advanced ER positive breast cancer.

In the first study, 24 postmenopausal patients were treated for three months with letrozole prior to surgery. A clinical response rate of 92% was seen in this group [101]. 15 of these patients who required mastectomy preoperatively were able to have breast conserving surgery after neoadjuvant treatment. A second group of 24 patients were treated with three months of anastrozole prior to surgery [104]. On ultrasound scanning, there was a 75.5% response to treatment after three months. Of the 17 patients who initially required mastectomy, 15 were able to have breast conserving surgery at the end of the three month period. A third group of 12 patients were treated with three months of neoadjuvant exemestane [105]. There was a >80% median reduction in tumour volume on clinical examination, ultrasound scan and mammography in this group. Out of 10 patients initially requiring mastectomy, eight were ultimately able to have breast conserving surgery.

All three aromatase inhibitors were shown to have a superior response rate to that seen with tamoxifen leading to increased tumour shrinkage and more patients being down staged to have breast conserving surgery. Additionally, in these small studies, the aromatase inhibitors were extremely well tolerated. Indeed, several studies have shown that patients taking aromatase inhibitors have similar or lower incidence of side effects than tamoxifen with fewer patients having to stop treatment because of adverse effects [87-89;94;106-112]. After such promising initial results from these pilot studies of neoadjuvant treatment with aromatase inhibitors, larger Phase III randomised studies were performed.

The PO24 study was a large randomised double blind multicentre study, comparing neoadjuvant letrozole with tamoxifen. In this study, 337 postmenopausal women with ER and/or PgR positive primary untreated breast cancer were randomised to receive 20mg tamoxifen or 2.5mg letrozole daily for four months [41]. The primary tumours were large at diagnosis (> T1) and were either inoperable (14%) or not suitable for breast conserving surgery. Baseline demographics and tumour characteristics were similar in the two groups.

Efficacy endpoint	Letrozole	Tamoxifen	P value
	(n= 154)	(n= 170)	
Clinical response	55%	36%	<0.001
Ultrasound response	35%	25%	0.042
Mammographic response	34%	16%	<0.001
Breast conserving surgery	45%	35%	0.022

Table 4: Results of the P024 trial comparing neoadjuvant tamoxifen and letrozole. Percentages refer to % of patients showing response or having BCS.

The results showed letrozole was significantly superior to tamoxifen in all three measurable endpoints, namely clinical response measured by palpation, ultrasound response and mammographic response (Table 4). There was also a significant difference in the number of patients who were able to be treated by breast conserving surgery (45% in the letrozole group vs 35% in the tamoxifen group, p= 0.022) after neoadjuvant therapy (Table 4).

The IMPACT trial (Immediate preoperative anastrozole, tamoxifen or combined with tamoxifen) compared 12 weeks of neoadjuvant treatment with anastrozole, tamoxifen or a combination of the two drugs in 330 post-menopausal women [113]. Biopsies were taken prior to starting treatment and after two and 12 weeks. Response rates as assessed by clinical examination were no different between the groups. The rate of breast conserving surgery was 44% for anastrozole compared with 31% for tamoxifen and 24% for the combined drugs. These results were not as impressive as the PO24 results for letrozole although there are several reasons why this might be the case. Firstly, the third arm in the IMPACT study resulted in fewer patients in each arm giving the study less power. The minimum tumour size for entry into IMPACT was smaller than for P024 so many patients enrolled in IMPACT may have had tumours which were suitable for BCS before starting neoadjuvant therapy. The required degree of oestrogen receptor expression was also lower in IMPACT. The treatment period in IMPACT was also shorter being three months compared to four in PO24. Another study, PROACT, also compared anastrozole and tamoxifen in the neoadjuvant setting. Importantly in this

study, patients could be given neoadjuvant chemotherapy in addition to their endocrine therapy during the three month treatment period [114]. 451 patients were randomised and objective response rates were higher with anastrozole than tamoxifen (37% vs 24%, p=0.03). In addition, significantly more patients were able to undergo breast conserving surgery in the anastrozole treated group (43% vs 31%, p=0.04).

One small study has compared neoadjuvant endocrine therapy using anastrozole with neoadjuvant chemotherapy using a regimen of doxorubicin and paclitaxel over a period of three months in 121 postmenopausal hormone receptor positive women [115]. This study showed response rates of 76% for chemotherapy and 90% for endocrine therapy with more breast conserving surgery being performed in the endocrine treated group (37% vs 21%). The same group presented a study comparing neoadjuvant exemestane with tamoxifen involving 151 postmenopausal women who were randomised to one or other drug for 3 months prior to surgery [116]. Exemestane was seen to perform better in terms of clinical response rate (76% vs 40%, p=0.05) and breast conserving surgery rate (37% vs 20%, p=0.05).

1.9 Optimal duration of neoadjuvant endocrine therapy

Standard practice with neoadjuvant chemotherapy has been to administer between three and six cycles prior to surgery. This approach provides enough time to distinguish between responders and non-responders and to achieve optimal tumour shrinkage preoperatively [117]. The optimal period of neoadjuvant endocrine therapy has never been investigated in detail.

One study in the Edinburgh Breast Unit gave neoadjuvant tamoxifen to 100 elderly patients (>70 years) with ER rich breast cancer (>20 fmol/mg cytosol protein) [118]. After three months, 72 patients showed tumour response (a greater than 25% reduction in tumour volume on ultrasound scan and only one patient had progressive disease. The remaining 27 patients continued on tamoxifen for a further three months. During this time, 18 patients continued with static disease, four responded but five patients had progressive disease. From these data, it can be concluded that if patients are not responding to an endocrine agent by three months then they are unlikely to respond if the drug is continued for longer. There is also concern that, if they continue on hormonal treatment alone, their disease may progress.

It would appear that three months is sufficient to demonstrate whether or not a tumour is responsive. However, maximal response may take considerably longer than three months so the optimal period of treatment depends on initial tumour size and the

purpose of the neoadjuvant therapy. If the purpose is to downstage the tumour to allow breast conserving surgery to be performed, this can be achieved in the majority of patients in three to four months.

1.10 Predicting response to endocrine treatment

The main purpose of all endocrine therapies is to improve survival. However, using survival as an end point takes many years because of the natural time course for breast cancer. Furthermore, survival is influenced by many confounding variables over time. It would be very useful to be able to accurately predict on an individual basis which tumours are likely to benefit from endocrine treatment so that a patient's treatment schedule could be individually tailored. One objective method of assessing tumour response to endocrine therapy is to leave the tumour in situ during systemic treatment. In this way, it would be possible to assess response to treatment and during treatment to obtain serial tumour samples to investigate the effect of the drug on different biological markers.

None of the classical clinical prognostic factors (eg grade, tumour size, lymph node status) predict sensitivity to endocrine therapy. ER is known to be the most important factor in determining response to endocrine treatment but it is not the only one, and not all patients who express ER, even when expression levels are high, respond to endocrine therapy.

1.10.1 Oestrogen and progesterone receptors and response to endocrine treatment

The initial step in the action of oestrogen is for it to bind to its receptor. Therefore, the presence or absence of oestrogen receptors in a tumour is an important indicator of which tumours will be endocrine sensitive. However, not all ER positive tumours respond to treatment. Although there is anecdotal data that some ER negative tumours appear to respond, this is probably related to ER positive tumours being classified as ER negative because of failures in the ER assessment technique.

60 – 75% of breast cancers are ER positive. A patient is more likely to have a tumour which is ER positive if it is low grade, if they are older and if they have become postmenopausal. Over recent years, there has been an increase in the percentage of tumours which are classified as ER positive. This could be due to an ageing population or alternatively, and more likely, to more sensitive hormone receptor assays.

It has been established clearly that response to neoadjuvant tamoxifen and aromatase inhibitors is related to ER status. However, even selecting an ER positive subgroup, only approximately 70% of patients respond to neoadjuvant tamoxifen [54;119]. Progesterone receptor (PgR) synthesis is oestrogen regulated and the level of PgR expression is theoretically an indicator of an intact oestrogen response mechanism and the functional integrity of the ER. PgR is synthesised by tumour cells that are stimulated

by oestrogens through an interaction with ER. This means that in theory PgR should be a better indicator of hormonal sensitivity than ER because, in some patients ER may be present but not functional. PgR can be detected in approximately two thirds of ER positive tumours. The incidence of PgR positivity also increases as the ER concentration increases.

Addition of PgR assessment increases accuracy in predicting response. Tumours that are both ER and PgR positive have higher response rates of between 66 and 83% than tumours that express only a single hormone receptor[119]. There is some evidence that small numbers of tumours, recorded as ER negative but PgR positive, respond to endocrine treatment. This is likely to be due to methodological problems with ER analysis resulting in occasional false negatives or a variant ER which is not detectable by the monoclonal antibodies used to detect ER. However, the ER that is present still stimulates PgR production.

During endocrine treatment, decreases in PgR expression within a tumour may be considered indicative of the anti-oestrogenic effect of treatment. Tamoxifen, a partial oestrogen agonist, has been shown to have dual dose-dependent oestrogenic and anti-oestrogenic properties. This is apparent in its induction of the progesterone receptor. With low doses, tamoxifen is potently oestrogenic and rapidly induces PgR over a 24-48 hour period. Indeed after four to six days the increase is four to ten fold. However, at high doses of tamoxifen, the induction of PgR is suppressed[120]. In the clinical setting it has been shown that neoadjuvant tamoxifen treatment increases PgR expression[121]. In

contrast, treatment with aromatase inhibitors decreases PgR expression over as short a period as 10-14 days.

1.10.2 Proliferation markers

After oestrogen binds to its receptor, it exerts action at a cellular level by stimulating proliferation. It may therefore be possible to accurately determine sensitivity to endocrine treatment by measuring changes in proliferation after a period of endocrine treatment. One proliferation marker which has been widely studied in breast cancer is Ki67.

Ki67 is a nuclear antigen that is expressed by cells in G1, S, G2 and M of the cell cycle but not during G0 [122]. Immunohistochemical staining for MIB1 (the monoclonal antibody to the Ki67 nuclear antigen) is therefore clinically useful as a marker of tumour proliferation. It is most frequently expressed in poorly differentiated tumours which have high rates of mitotic activity. High levels of proliferation are associated with early recurrence of breast cancer after mastectomy [123]. However, the amount of staining seen is independent of tumour size, lymph node status and ER expression [123].

Ki67 levels in normal breast tissue have been shown to be unaffected by neoadjuvant endocrine therapy with letrozole [124]. In this study, 32 women without active breast disease were recruited to a study where they were given three months of treatment with letrozole (2.5 mg/day) Core-cut biopsies from the breast were collected before and at the end of treatment. There was no significant change in the proliferation marker Ki67 or oestrogen receptor in breast epithelial cells with treatment.

Proliferation when assessed using Ki67 has been shown to decrease by 79% in patients after treatment with tamoxifen for as short a period as 10-14 days [125]. This was shown to correlate with subsequent clinical response in that one small study. In this study all patients who responded to tamoxifen showed a decrease in Ki67 at 14 days. In those who did not respond, any changes were small, with a median value close to zero. However, by eight weeks, the observed reductions in Ki67 no longer correlated with response. Other studies have failed to show that a drop in proliferation correlates with clinical response.

Proliferation has also been shown to drop early during treatment with aromatase inhibitors. Studies continue to investigate the effects of AIs on biological markers early in neoadjuvant treatment to see if reduced proliferation does correlate with subsequent clinical response.

1.10.3 Epidermal growth factor receptor (EGFR) and HER2 (Erb B2)

The ErbB family of cellular type I receptor tyrosine kinases (TKs) plays a central role in normal cell proliferation, survival, and differentiation in a variety of tissues. Ligand binding to the epidermal growth factor receptor (EGFR, ErbB-1) results in receptor activation. ErbB-2 (HER-2) activation results in activation of signalling pathways involved in cell proliferation, survival, and transformation. Over expression of the EGFR or ErbB-2 receptors results in cell transformation and is associated with poor clinical outcome in a number of malignancies [126;127]. The HER2 gene is amplified and over expressed in 25-30% of breast cancers. It is more likely to be over expressed in ER negative tumours than ER positive tumours where approximately 10% of tumours have an amplified HER2 gene. The potential roles of the EGFR and ErbB-2 receptors in tumour cell proliferation and survival have led to the development of monoclonal antibodies that inhibit the receptors. This includes the well publicised trastuzumab (Herceptin®) which is a monoclonal antibody to the ErbB-2 receptor.

EGFR is present in almost half of all clinical breast tumours. In breast cancer tissue, excess EGFR expression is associated with increased tumour proliferation, and a poor prognosis. There is an inverse relationship between ER and EGFR expression with over expression of EGFR determining a highly malignant potential. It is thought that EGFR expression may represent the progression of a cell towards oestrogen independence and

high levels of expression have been shown in tumours relapsing on tamoxifen. Expression of EGFR prior to treatment in locally advanced and metastatic breast cancer has been shown to predict tumours which are less likely to respond to primary therapy with anti-oestrogens.

In experimental models, human epidermal growth factor receptor-2 (HER-2) amplification has been shown to correlate with oestrogen independence and tamoxifen resistance in ER positive human breast cancer cells. Some reports suggest an association between HER-2 positivity and hormone independence in breast cancer patients.

There has been little published work on the relationship between aromatase inhibitors and Erb B-1 and Erb B-2 over expression. Small non-randomised studies in Edinburgh showed that the response rate to neoadjuvant anastrozole was similar in both erb B1/B2 positive patients and negative patients [128]. However, differences in response between letrozole and tamoxifen in the PO24 study were largest in the subgroup of tumours that was ER and or PgR positive and were also positive for Erb B-1 and/or Erb B-2 (88% for letrozole vs 21% for tamoxifen) [129]. It has been hypothesised that these patients are resistant to tamoxifen but respond to aromatase inhibitors. This could explain why aromatase inhibitors appear to have improved efficacy in direct comparison with tamoxifen. Assessment of HER2 status for prognosis and treatment of breast cancer patients can be performed by immunohistochemistry and/or fluorescence in situ hybridization (FISH).

1.11 Neoadjuvant endocrine therapy study models

There have been two main study models described to investigate the effects of endocrine agents on breast cancer in vivo.

The preoperative model involves giving patients preoperative endocrine therapy in the 14-21 days between diagnosis and surgery (Figure 4). Tissue is taken at the time of diagnosis and further tissue is obtained from the same tumour during definitive surgery. Studies have shown that without any intervening treatment the tumour remains stable with no changes in hormone receptor expression or proliferation. This allows comparison of tumour tissue before and after exposure to agents such as different endocrine therapies, with the certainty that changes are as a consequence of the drug therapy. A limitation of this model is that information on clinical response is not available to correlate with the biological changes which occur as a result of the drug treatment. Figure 4 summarises the preoperative model where patients have an initial diagnostic biopsy and then 14-21 days later undergo surgery. Patients are given drug treatment between these two time points and changes in the tumour can be correlated with the drug given.

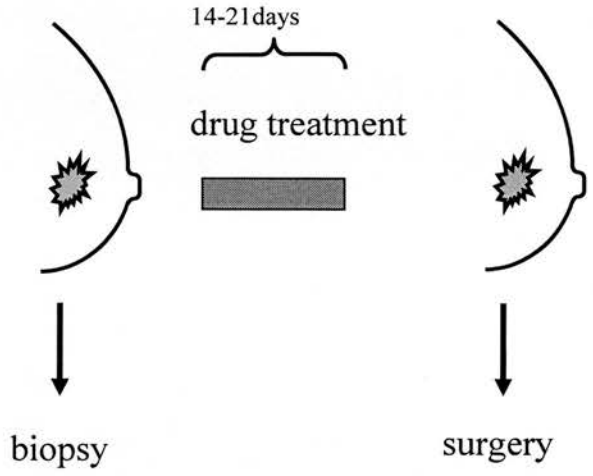

Figure 4: Preoperative model of neoadjuvant endocrine therapy

The other model system involves treating patients with neoadjuvant endocrine therapy for between three and four months (Figure 5). Tissue can be collected at different time points during the study period. Clinical response is assessed by both clinical examination and imaging over the treatment period. This model has the advantage that clinical response can be correlated with pathological and biochemical changes in individual patients and their tumours.

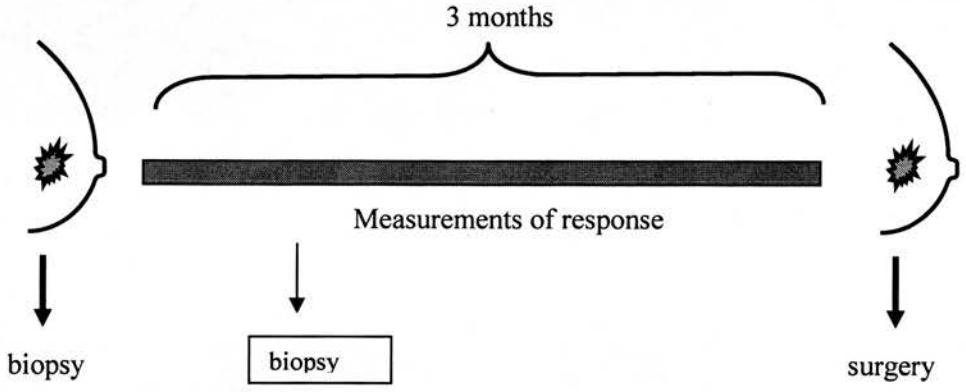

Figure 5: Three month model of neoadjuvant endocrine therapy

1.12 Assessing clinical tumour response to neoadjuvant therapy

It is important to be able to assess accurately the degree of response to neoadjuvant treatment in order to be able to assess its effectiveness. The natural course of breast cancer untreated is to grow in size, but the rate of growth is variable with a median doubling time of approximately 60 days. It is however rare for a tumour to regress spontaneously and therefore sustained reduction in tumour size is likely to be as a result of therapy. It remains a challenge to accurately measure tumour size over time.

Potential methods of serial assessment are performing clinical examination and calliper measurements, mammographic measurements or ultrasound measurements. Previous studies have shown that ultrasound response is the modality which corresponds most closely with pathological response [130].

1.12.1 Clinical measurements

Clinical measurements by calliper are notoriously inaccurate, being prone to large degrees of inter-observer variability and influenced by factors such as tissue oedema and patient obesity [131;132]. The method used is indirect with the tumour being measured through the skin by palpation and using callipers. The measurement can also be influenced by the depth of the tumour within the breast tissue. It has been demonstrated that two observers may produce significantly different measurements for the same inoperable breast cancer. [133]. However, it has been shown that if the same observer remained consistent in their method, changes in lesion size were very similar over time. Only those lesions which were discrete and easy to measure gave reproducible results in this study and observers had to remain blind to their previous measurements. The false positive rate of this method is also reduced if two or more successive measurements show a consistent change. Therefore, the optimal method of assessing response by clinical measurement alone is for one observer to make repeated serial measurements over a period of time.

1.12.2 Mammographic measurements

Mammography can be used as an objective method of assessing response of breast cancers to treatment but it is also not without concerns. Frequent repetition of mammography is not advisable because of the dose of radiation involved. In some elderly women, who often have additional comorbidity, it can be technically difficult to obtain mammograms because of immobility. Additionally, in certain types of tumour such as lobular carcinoma, the margins are indistinct thus making measurement and comparisons between mammograms difficult and sometimes inaccurate.

Comparisons have been made between assessing clinical and mammographic response to systemic therapy. In one study, physical examination overestimated the overall response rate in 22.9% of cases while the converse was true in 8.6% of cases [134]. However, both clinical examination and mammography have the problem of only being able to assess the tumour in two dimensions.

1.12.3 Ultrasound measurements

It has been shown that ultrasound scanning correlates most closely with pathological size when compared with clinical examination and mammography [135]. The same study showed that inter- and intra-observer variation was less than 10%. Ultrasound has the advantage of being able to assess tumours in three dimensions thus allowing depth to be added to the volume calculation.

Breast ultrasound is simple to perform and cheap. It can be performed repeatedly as it does not involve any radiation dose and a printed permanent record of the measurements can easily be obtained. For these reasons, ultrasound scanning has become the method of choice for serial assessment of response to neoadjuvant endocrine treatment in breast cancer.

1.13 Aims

Neoadjuvant endocrine therapy is being used increasingly as primary treatment in very elderly or infirm patients in order to avoid the need for surgery. It is also being used to downstage tumours, since it allows inoperable tumours to become operable and allows tumours which would have required mastectomy to be suitable for breast conserving surgery. However, concern has been expressed that women who fail to respond to neoadjuvant treatment have a delay in effective loco-regional treatment which may have an impact on their survival.

The ability to predict which patients respond to treatment would have the benefit of allowing selection of those likely to respond to neoadjuvant treatment. Others not likely to respond would change to more effective local or systemic treatment. Studying patients with their primary tumour left in situ during systemic drug treatment allows clinicians to make an accurate estimation of response to therapy. In parallel, access to serial cores of the same tumours during treatment allows measurement of biological factors that may predict for response to treatment. Additionally, this knowledge should aid understanding of the mechanisms of action of, and help explain resistance to, these treatments. Taking serial fresh frozen tumour samples allows expression of genes to be assessed using microarray work in order to look at gene expression in parallel with clinical response.

This thesis therefore aims to characterise the response of primary breast cancers to neoadjuvant endocrine treatment with letrozole and to compare the biological changes in tumour phenotype over the initial 14 days of treatment and after 3 months. The objectives were:

- to further characterise the clinical response to primary systemic endocrine therapy in breast cancer in the setting of a clinical trial;

- to determine whether it is possible to identify biological markers of tumour phenotype that can be used to predict subsequent clinical response to neoadjuvant treatment with 2.5mg letrozole for three months;

- to determine the effects of treatment on biological markers after 10-14 days and after three months and investigate whether these changes correlated with clinical responses seen over that period; and

- to identify potential markers of resistance to endocrine therapy.

In order to address these aims, the letrozole audit was carried out as a three month neoadjuvant study which treated 137 postmenopausal patients with locally advanced breast cancer with 2.5mg letrozole daily. Samples of tumour were taken both at

diagnosis and after 12 weeks and, in 72 patients, additionally at 10-14 days. Serial clinical tumour measurements were made at diagnosis and after six and 12 weeks using clinical examination and ultrasound scan. Mammography was performed, both at original diagnosis and after three months.

Firstly the clinical response of tumours to treatment with letrozole over three months will be discussed. Then the effects of treatment on biological responses to therapy will be reviewed. In conclusion, there will be a discussion about the significant findings and the prospects for further research.

Section 2: Materials and Methods

2.1 Ethical approval

Prior to commencement, this research was approved in advance by the Lothian Research and Ethics Committee and the NHS Trust Research and Development Department. All studies were discussed in advance at the local breast cancer multidisciplinary meeting and all patients who were suitable to be approached regarding taking part in this study were discussed by the whole multidisciplinary team in advance. This involved discussion between pathologists, medical and clinical oncologists, surgeons, radiologists, breast care nurses and research staff.

2.2 Patient populations

All patients were recruited from the new patient clinics at the Edinburgh Breast Unit based in the Western General Hospital in Edinburgh. Patients were given an information booklet about the study (Appendix 1) in addition to discussing it with medical and nursing staff before deciding about whether or not they wanted to participate.

Patients were given the option of agreeing to allow extra samples to be taken at the time of diagnostic core biopsy and stored for research purposes. They completed a consent form which had been approved for this purpose by the local ethics committee (Appendix 2). Additional core biopsies at 10-14 days were optional and if patients were happy to allow these to be taken they completed a standard hospital consent form for this purpose.

Core biopsies were performed in the outpatient clinic under local anaesthesia and were well tolerated. The biopsies were routinely assessed in the pathology department for ER status using immunohistochemistry. The patient's ER status was used to select those suitable for neoadjuvant endocrine trials. Patients with a tumour that was ER rich (defined as an Allred score of 6-8) were most likely to benefit from endocrine therapy. When patients returned to the clinic to get the result of their core biopsy neoadjuvant protocols were discussed with them, if considered appropriate, as part of their treatment plan. All aspects of the patient's management, including potential trial involvement,

were discussed by the local multidisciplinary team. This ensured that only appropriate patients were selected and that there was unanimous agreement that the patient was suitable for the proposed treatment. A copy of the multidisciplinary team's decision was recorded in the patient's case notes. Patients who agreed to take part in a trial signed the appropriate consent form for that trial.

Clinical follow up during the study period was performed by, or organised by, the author and Mr Dixon, consultant surgeon with the assistance of Miss Renshaw, senior research nurse.

2.3 Patient selection

Theoretically, any post-menopausal woman with locally advanced breast cancer being considered for surgery as primary treatment could be enrolled to receive preoperative endocrine therapy, regardless of clinical stage, providing that their tumour was oestrogen receptor positive. In practice, this meant that patients with distant metastatic disease were excluded because surgery was not usually part of their primary treatment. Patients with severe renal or hepatic impairment were also excluded, as were patients who were considered to be at risk of transmitting HIV or Hepatitis B or C. Anyone unable to give informed consent was automatically excluded.

Patients considered eligible for the neoadjuvant study model formed a specific group. The majority had large operable or inoperable advanced tumours. These would potentially derive benefit from tumour shrinkage and tumour downstaging, following successful primary endocrine therapy. These patients were predominantly elderly with a tumour larger than 3cm clinically (2cm on imaging) which was oestrogen receptor positive on core biopsy. The majority of patients involved in the study had surgery at the end of the treatment period but some, who were considered unfit or who declined to have surgery, continued on primary endocrine therapy if their tumour showed evidence of clinical response. Some younger post-menopausal patients with large primary tumours were enrolled onto the three month neoadjuvant study because it was felt that they might benefit from having their proposed surgery downscaled from mastectomy to breast conserving surgery.

2.4 Methods of assessing tumour response

Patients enrolled on the three month neoadjuvant therapy trial had their tumour response assessed using the following methods.

2.4.1 Clinical examination

Examination was carried out using bidimensional caliper measurement, either by the author or Mr Dixon, consultant breast surgeon. The size of the primary tumour was determined by measuring two different tumour diameters at 90^0 apart. Tumour size was then determined by multiplying the longest diameter by the greatest perpendicular diameter (bidimensional measurements). Tumour volume was assessed using the formula;

$V = \frac{D^3 \times \pi}{6}$ where V is the volume and D is the mean diameter

Assessment was also made of any change in the appearance of the local tumour eg tumour ulcerating or re-epithelialising (Figure 6).

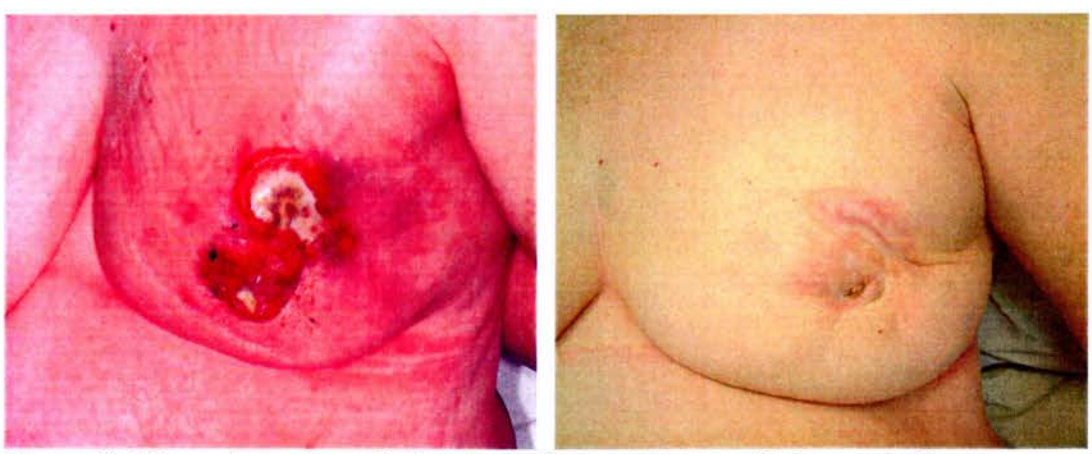

Figure 6: Ulcerating tumour before and after treatment with letrozole for three months

2.4.2 Ultrasound measurements of tumours

A Honda convex scanner model HS-2000 with a linear array probe was used to perform all the ultrasound scan measurements. All scans and measurements were performed by one of the same two investigators (the author and Mr Dixon). The probe was held perpendicular to the skin surface and moved over the tumour until a maximum diameter was visualised. The tumour was then visualised in two different planes, and in each view, bidimensional measurements were made across the widest part of the tumour (Figure 7). The response to treatment was also assessed on the basis of tumour volume changes which were calculated using the formula for the volume of an ellipsoid [135]. This uses two different diameters measured at 90^0 intervals and, additionally, the thickness of the primary breast lesion (tridimensional measurements).

$$V = \frac{D^2 \times d \times \pi}{6}$$

where V is the volume, D is the mean of diameters parallel to the skin surface, and d is the mean thickness as measured by the ultrasound machine's electronic calipers

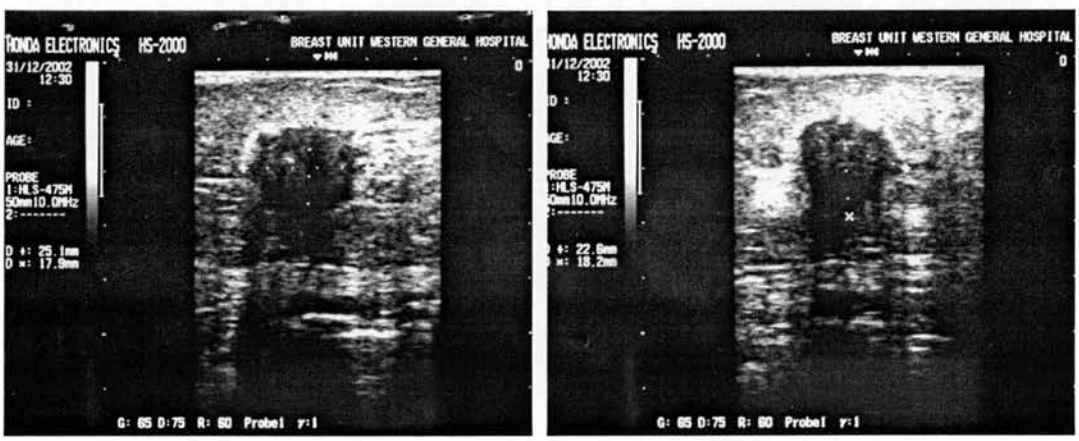

Figure 7: Measurements being taken of tumour on ultrasound scan

During training in this technique, the same tumours were scanned and measured blindly by the author and Mr Dixon (JMD), consultant breast surgeon. There was less than a 10% inter-observer error similar to that previously reported by Forouhi [135].

2.4.3 Mammographic measurement of tumours

Two-view mammography (oblique and craniocaudal views) was performed at the start and end of the study period (Figure 8). The largest tumour diameter and the diameter at 90° to the axis were measured. The mean mammographic diameter and tumour volume were calculated using the following formula;

$$V = \frac{D^3 \times \pi}{6}$$ where V is the volume and D is the mean diameter

Bidimensional assessment and percentage change in volume were used to assess response to therapy, comparing tumour area at the start of treatment with that after three months.

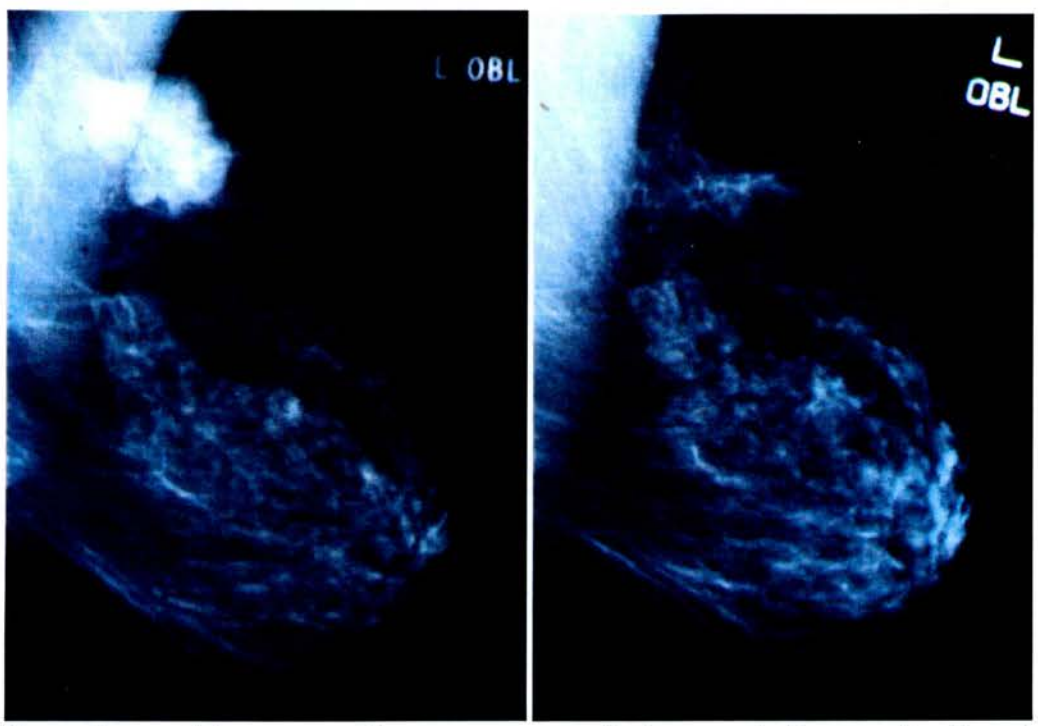

Figure 8: Mammographic appearance of tumour before and after letrozole treatment, showing a marked reduction in tumour size and density

2.4.4 Pathological assessment

Pathological response was assessed in those patients who had surgery at the end of their period of neoadjuvant treatment. Bidimensional tumour size was measured by the pathologist on the excised specimen. Both macroscopic and microscopic measurements were carried out to determine the extent of the invasive tumour. Determining pathological size accurately allowed direct comparisons to be made with final tumour size as assessed by clinical examination with calipers and mammography and ultrasound scan. Axillary lymph nodes were also examined histologically for the presence of metastatic tumour.

Histopathological features of the tumours after three months treatment were compared with the diagnostic and 10-14 day core biopsy specimens using Haematoxylin and Eosin (H&E) stained sections. Tumour morphology was judged by comparing changes in cellularity and fibrosis. The grading was also scored by looking at tubule formation, nuclear pleomorphism and the frequency of mitosis. This allowed an overall tumour grade to be calculated (Appendix 3). This method is described in more detail by Elston and Ellis [136].

2.4.5 Evaluating tumour response

Modified World Health Organisation (WHO) criteria were used to evaluate tumour response as follows:

Complete response (CR):	no measurable tumour
Partial Response (PR):	reduction in tumour bidimensional area ≥50% from pre-treatment size.
Minor response (MR):	reduction in tumour bidimensional area ≥25% and <50% from pre-treatment size.
No change (NC):	<25% decrease or <25% increase in tumour area from pre-treatment size.
Progressive disease (PD):	25% or more increase in tumour area from pre-treatment size.

2.4.6 Assessment of side effects

At each visit to the clinic, all neoadjuvant patients were asked about any side effects of treatment. Patients were asked to contact study personnel in addition to their GP if they noted any serious side effects or if they were considering stopping treatment because of side effects.

2.4.7 Survival

The progress of all patients was followed up to obtain information on disease free survival, distant disease free survival and overall survival. Survival data were calculated in May 2006 but follow up of these patients is still ongoing. Recurrences of tumour in the chest wall, ipsilateral breast or axillary nodes were classified as local recurrences. Any other recurrences were classified as distant recurrences. Survival was classified as survival with or without evidence of recurrent disease. The follow up period was calculated from the date of beginning drug treatment to May 2006, the date on which data was analysed.

2.4.8 Definitive locoregional surgery

At the time of starting treatment, the type of surgery that would have been required to remove the tumour was determined by Mr Dixon who operated on all study patients who had surgery. Whether the patient required to have a mastectomy, or a wide local excision was noted in addition to an axillary node clearance or sample. On completion of systemic therapy, the majority of neoadjuvant patients had surgery to remover their tumour. The type of surgery actually performed was recorded and compared with the surgery that would have been required prior to neoadjuvant treatment. The facilitation of local conservative treatment is an important outcome in neoadjuvant therapy, especially in breast cancer where there is significant psychological morbidity associated with mastectomy. However, it remains to be confirmed whether this will have any long term impact on breast cancer recurrence and survival.

2.5 Sampling techniques

In both studies several techniques were used to collect and process tissue samples.

2.5.1 Core biopsy

An area of skin overlying the tumour was prepared with betadine solution. 20ml of 1% lignocaine with a 1:200000 adrenaline solution was then infiltrated under the tumour. The local anaesthetic and adrenaline was left to become effective for at least 10 minutes. A 15 bladed scalpel was used to make a small incision in the skin over the palpable tumour. A Bard Max Core Disposable Biopsy Instrument with a 14G needle was used to take serial biopsies through the same skin incision. Some of the biopsies were placed in formalin to allow later histological examination and others were placed in round bottomed cryogenic vials and immediately fresh-frozen in liquid nitrogen. At least four cores were taken for diagnostic purposes and wherever possible a further four were fresh-frozen in liquid nitrogen for later research. After biopsy, direct pressure was applied over the site to reduce bleeding, and a dressing was then applied. Patients were advised to leave this in place for 24 hours. The availability of mammatome biopsies allowed larger samples of tissue to be taken under ultrasound guidance from some larger tumours (see figure 9). This device used an 11 or 8 gauge core needle (see figure 10) and vacuum suction assistance to remove larger pieces of tissue than a conventional core gun.

Figure 9: Mammatome biopsy being performed

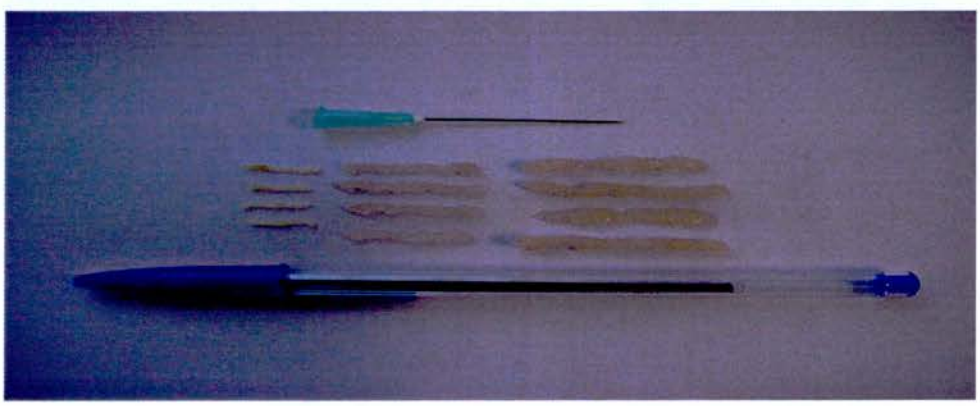

Figure 10: Comparison of the size of core biopsies (left) with mammatome biopsies – rows are 18 gauge core biopsy and 11 gauge mammatome biopsies

It was important that the fresh samples were frozen in liquid nitrogen as quickly as possible (within a few minutes of being taken) to allow future analyses. This required a container of liquid nitrogen to be available adjacent to the clinic where the samples were taken.

2.5.2 Tissue collection from theatre specimens

Further tissue samples were taken at the time of surgery and both fresh-frozen and formalin-fixed tissues were obtained. In order to do this, either a pathologist was available to take a piece of fresh tissue from the excised specimen or one of the surgeons took core biopsies from the sample or excised a small part of the tumour as soon as possible after the specimen was removed. The sample was then fixed in formalin and paraffin sections were obtained after routine processing.

2.5.3 Storage of fresh-frozen specimens

Fresh-frozen samples were stored in liquid nitrogen in 2ml round-bottomed cryogenic vials. Relevant identification details were marked clearly using a suitable cryogenic pen. Patient's details were encoded to anonomise samples. Details of all samples collected, together with the date the biopsy was performed and details of the study the patient was enrolled in, were recorded in a detailed log to allow accurate sample identification at a later date. Immediately after samples were taken and placed into the cryogenic vials, they were put into liquid nitrogen prior to being transferred to the tissue bank. If samples taken from wide local excision or mastectomy specimens could not be taken immediately, specimens were placed on ice whilst awaiting specimen radiology or sampling by a pathologist.

2.5.4 Processing of paraffin embedded specimens

As soon as possible after being taken in clinic or theatre, formalin-fixed tissue samples were placed directly into 4% buffered formalin. They were then taken to the pathology department for processing and paraffin embedding.

2.5.5 Preparation of blocks
(Specimen Automated Processing Protocol)

Tissue samples were fixed in 4% buffered formalin for 24 hours before processing. The tissue was then placed in a cassette and processed on a Vacuum Infiltrated Processor (VIP, Tissue Tech VIP, E300 series, Miles Inc) overnight. Treated tissue was then placed in a mould and covered in paraffin wax. The detailed protocol is described later (see appendix 4).

2.5.6 Cutting paraffin sections

Paraffin blocks were first cooled on ice. Using a microtome, 4μm thick sections were cut from the blocks. Sections were floated on a water-bath before being placed on Superfrost glass slides. The sections were fixed to the slides by incubation at 37°C overnight.

2.5.7 Analysis of tissue samples

Fixed tissue blocks can be used for a wide range of analyses. The preliminary staining that was performed on study tissue samples included oestrogen receptor (ER), progesterone receptor (PgR) and a marker of proliferation (Ki67). EGFR and erbB2 were also assessed on the majority of samples.

Fresh tissue was collected from patients since the end-points involved enzyme activity or the analysis of genetic material by reverse transcription using the polymerase chain reaction (RT-PCR) or microarray. The morphology of core biopsies on frozen section was assessed before the material was analysed. This ensured that sufficient malignant tissue was present within the biopsy for analysis. Mammatome biopsies were particularly useful for this purpose as more tissue was available. Frozen sections were kept of each sample submitted for gene array analysis to ensure that there was adequate tumour in the cores before they underwent this costly investigation. Tissue morphology was often slightly damaged when they were stored in liquid nitrogen. In some samples, it was necessary to microdissect specimens to ensure the sample contained enough malignant tissue.

Work is ongoing involving the fresh tissue samples from patients in the studies described but the results are outwith the scope of this thesis.

2.6 Staining techniques for paraffin sections

All paraffin sections were first H&E stained to ensure that there was sufficient invasive cancer in the specimen to allow analysis. The method is described later (See appendix 5).

2.6.2 Immunohistochemistry

Similar immunohistochemical methods were used to detect a variety of biological markers. Tissue from the same patient at different time points was stained during the same run to minimise potential inter-run variation. 4μm sections were cut from paraffin embedded tumour blocks. The sections were dewaxed and placed in 3% H_2O_2 to block endogenous peroxidase activity. The sections were placed in antigen retrieval buffer (citrate or EDTA) in a pressure cooker and microwaved for six minutes (at full power in a domestic 800 watt microwave oven). They were then rinsed in distilled water and tris buffered saline (TBS). Protein block (DAKO: X0909) was added to each section to stop endogenous peroxidase activity. Sections were rinsed with TBS and the primary antibody was added for the desired time. Sections were again washed with TBS before En-Vision chemmate solution (DAKO kit:K5007) was added. After washes in TBS, DAB Chromogen solution (DAKO kit: K5007) was added to visualise the staining. After washing in distilled water, copper sulphate was applied to enhance the staining. Sections were finally counterstained with haematoxylin before being taken up through graded alcohols to absolute alcohol. The slides were mounted from xylene then coverslipped. Positively stained structures appeared brown when viewed under direct light microscopy (Figure 11). Full details of the immunohistochemical technique are given later (See appendix 6).

2.6.3 Oestrogen and progesterone receptors

Oestrogen and progesterone receptor status was assessed by immunohistochemistry, using the envision technique after microwave antigen retrieval using 6F11 (Dako) for ER-α and PgR636 (Novacastra) for PgR. The method is described later (See appendix 6). Positive control slides were used with each rack of slides in each run.

They were Allred scored [137] as described below. This adds the scores of proportion and intensity of staining according to the following criteria.

Proportion of cells stained	Score	Intensity of staining	Score
0	0	None	0
< 1%	1	Weak	1
1 – 10%	2	Intermediate	2
11 - 33%	3	Strong	3
34 – 66%	4		
> 66%	5		

The Allred score is calculated by adding the proportion score to the intensity score (P + I). It therefore ranges from 0 to 8 although there can be no Allred score 1.

2.6.4 Allred scoring

The Allred score for ER has been shown to correlate with the response to endocrine treatment in breast cancer [137]. This method is easy to learn and highly reproducible[137]. With minimal training, the pathologists involved in the study by Harvey et al were in agreement in discriminating positive from negative tumours in 99% of cases. Allred scoring has become the standard scoring of ER and PgR in the majority of pathology laboratories. Harvey et al showed that the optimal cut off point was an ER score greater than 2. This means that even patients scoring 3, which corresponds with as few as 1-10% of weakly staining positive cells, had a significantly improved outcome with endocrine therapy compared with those who had lower scores.

a) b)

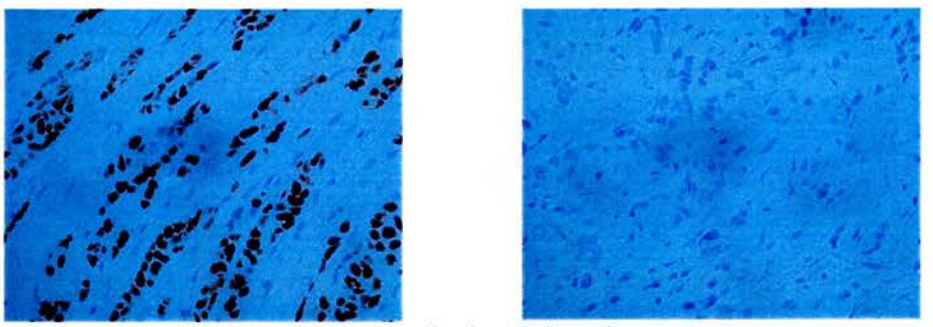

Figure 11: Immunohistochemical staining for PgR a) pre-treatment and b) 14 days after treatment with letrozole

2.6.5 Training in Allred scoring of immunohistochemically (IHC) stained slides

Professor T J Anderson (TJA), consultant pathologist and Professor W R Miller (WRM), consultant in experimental oncology at the Western General Hospital in Edinburgh trained the author and other investigators to assess and Allred score ER and PgR in primary breast cancers by IHC on formalin-fixed paraffin-embedded tissue sections. Initially, 100 slides were scored independently by the author and TJA or WRM. Results were in complete agreement in 67% of cases and varied by only one IHC score in the remaining 33% of cases. This showed a high rate of concordance (weighted kappa 0.96 for proportion and 0.56 for intensity, see tables 4 and 5 and appendix 7).

Proportion	Scorer 2				
Scorer 1	1	2	3	4	5
1	2	1	0	0	0
2	0	0	0	0	0
3	0	0	1	0	0
4	0	0	0	2	0
5	0	0	0	0	44

Table 4: Inter-observer variation in proportion of staining cells as component of Allred scoring ER/PgR stained slides. Kappa = 0.91 (95% CI [0.74, 1.00]). Weighted kappa = 0.96 (95% CI [0.90, 1.00])

Intensity	Scorer 2		
Scorer 1	1	2	3
1	1	1	0
2	2	18	3
3	0	7	18

Table 5: Inter-observer variation in intensity of cell staining as component of Allred scoring ER/PgR stained slides. Kappa = 0.53 (95% CI [0.31, 0.74]). Weighted kappa = 0.56 (95% CI [0.36, 0.76])

To further reduce the likelihood of potential inter-observer variation, two investigators scored every study slide. If results varied, the slide was re-examined jointly by both investigators and a decision was made on the final agreed Allred score. This was only required in a minority of cases.

The same slides were also scored at different time points by the same investigators. Again concordance was high (weighted kappa 0.88 for proportion of staining cells and 0.91 for intensity of staining) with investigators always being at least within one Allred score of their previous score (see tables 6 and 7 and appendix 8).

Proportion	Scorer 2				
Scorer 1	1	2	3	4	5
1	0	0	0	0	0
2	0	0	0	0	0
3	0	1	0	2	0
4	0	0	0	2	0
5	0	0	0	0	17

Table 6: Intra-observer variation in proportion of staining cells as component of Allred scoring ER/PgR stained slides. Kappa = 0.81 (95% CI [0.52, 1.00]). Weighted kappa = 0.88 (95% CI [0.74, 1.00])

Intensity	Scorer 2		
Scorer 1	1	2	3
1	2	0	0
2	0	13	0
3	0	1	4

Table 7: Intra-observer variation in intensity of cell staining as component of Allred scoring ER/PgR stained slides. Kappa = 0.90 (95% CI [0.70, 1.00]). Weighted kappa = 0.91 (95% CI [0.72, 1.00])

2.6.6 Cell proliferation- Mib1 staining

Ki67 antigen expression was assayed by measuring the binding of a mouse monoclonal antibody, Mib1 (Dako), to the Ki67 nuclear antigen using EnVision immunohistochemistry as described later (See appendix 9). The slides were scored using the technique described on page 103 and appendix 6.

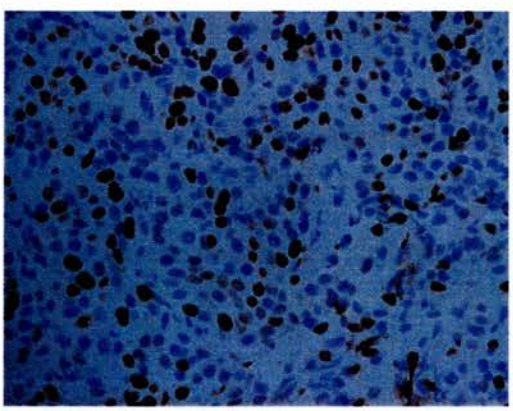

Figure 12 a) at baseline

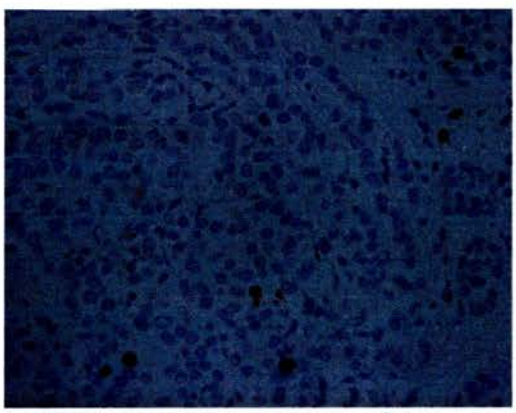

Figure 12b) after 14 days of drug treatment

Figure 12: Assessing proliferation using immunohistochemistry to detect the Ki67 antibody. The brown staining cells are those proliferating. It can be clearly seen that there are far fewer proliferating cells after 14 days of drug treatment than before.

Sections of appendix were used as the positive control slides with every run (see Figure 13).

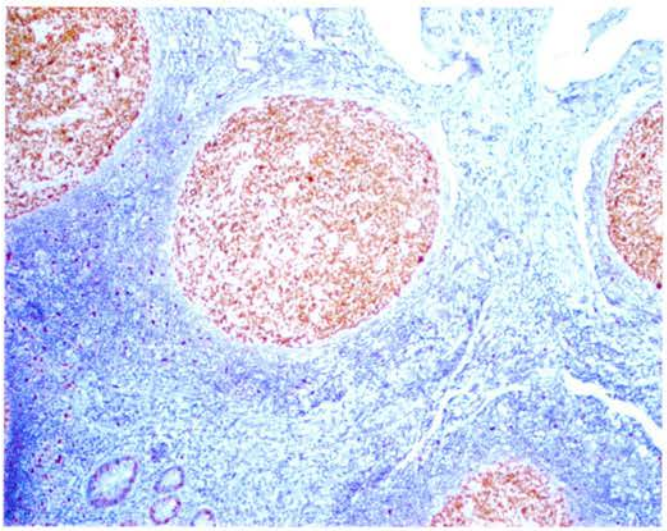

Figure 13: Control slide (appendix) for assessing proliferation using Mib

2.6.7 Scoring proliferation

Several methods have been described to score proliferation using Ki67 staining. Three different methods were assessed in this study in order to identify which one which was the most accurate and reproducible.

In the first method, the percentage of positively staining cells in several fields was assessed by a consultant pathologist (TJA). An estimate of the proportion of cells staining across the slide was then made. In the second method, a graticule incorporating a single line bisecting the field was used. At x40 magnification, the number of negatively staining nuclei being transected by the line was counted (n). The formula for area was used to estimate the cell count ($A=\pi r^2$) where n was equal to the diameter of the microscope field, n/2 =r and A equals the estimated total cell count. The positive cells in the field were also counted. This was repeated in 10 fields. The technique is described in detail by Simpson et al [138].

In the third method, sections were examined under a light microscope using x40 objective and a 10x10 eye piece incorporating a graticule. 10 of the 100 squares were marked randomly and the negative cells in these squares were counted. This number was multiplied by 10 to estimate the total number of negative cells in the field. Then all the positive cells in the field were counted. New fields were counted until the total cell count exceeded 1000 cells. Ki67 positive nuclear staining cells were then calculated as a

percentage of the total cells. This method has also been previously described [139]and is now widely used for the assessment of Ki67 using Mib antibody.

The results of the three methods of scoring were compared (Table 8).

Patient number	Pre-treatment			After 10-14 days			After three months		
	A	B	C	A	B	C	A	B	C
6	15	11.82	7.51	1	1.28	0.45	1	0.1	0
7	25	19.76	12.16	1	2.39	1.84	1	0.39	0.64
8	30	35.09	24.09	10	9.76	3.79	5	15.54	4.02
10	5	5.02	9.83	1	1.7	2.4	1	0.46	0.08
12	15	4.62	8.15	1	3.49	1.64	1	9.46	5.25
15	25	19.2	21.8	1	0.6	1.3	1	0	0
17	5	4.6	6.34	1	1.81	3.91	5	0.06	0.25
18	40	33.69	19.74	1	1.38	5.71	5	10.21	15.86
23	15	16.2	4.98	35	7.03	4.55	10	7.51	1.18
24	25	15.13	10.8	5	2.81	1.75	1	1.04	2.29
25	15	19.08	14.48	5	2.4	7.26	1	0.07	5.32
26	25	23.72	19.73	10	6.18	1.47	1	8.11	0.93
27	25	32.21	22.06	1	2.23	7.74	1	0.8	3.21
34	30	27	17.57	10	10.49	8.93	5	3.1	0.8
38	20	22.49	15.78	5	17.48	4.33	10	28.54	8.19
43	10	29.5	10.08	5	7.4	3.59	1	11.5	1.98
44	30	108	16.5	5	10.2	2.89	1	6.4	2.31
50	35	34.2	19.76	10	15.8	3.49	25	18.29	15.79
51	40	34.1	15.53	1	31.8	13.4	20	40.4	16.8
53	5	8.9	17.85	1	8.6	2.84	1	8.3	0.68
57	30	24	1.64	15	17.2	24.78	25	25.9	15.54
58	25	23.4	22.32	5	15	2.67	0	0	0.84

Table 8: Assessing different scoring methods to assess proliferation

Method A = estimated proportion of cells staining (no direct counting of cells).

Method B = scoring technique as described by Simpson et al.

Method C = scoring technique as described by Johnston et al.

To assess which method was most accurate and reproducible, they were repeated by two investigators blinded to each others results. The third method described above proved to be the most accurate and reproducible and was therefore the method of counting cells used in these studies.

Proliferation in these studies was assessed by the author and one of the other investigators, Miss Sharon White. Professor T J Anderson, consultant pathologist re-examined a selection of cases from the study and concordance with scoring was high both between observers (Pearson's correlation $r = 0.97$, $p<0.001$) and with one investigator repeating scoring of the same slides at a different time (Pearson's correlation $r = 0.95$, see appendices 9 and 10 and figures 14 and 15).

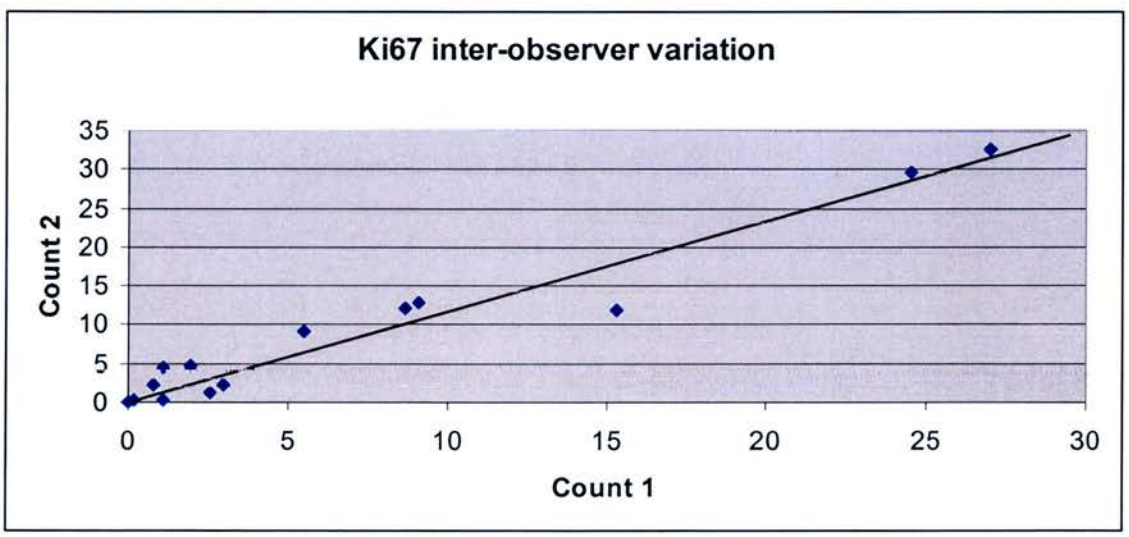

Figure 14: Inter-scorer correlation in scoring same slides. Pearson's correlation $r = 0.97$ ($p<0.001$)

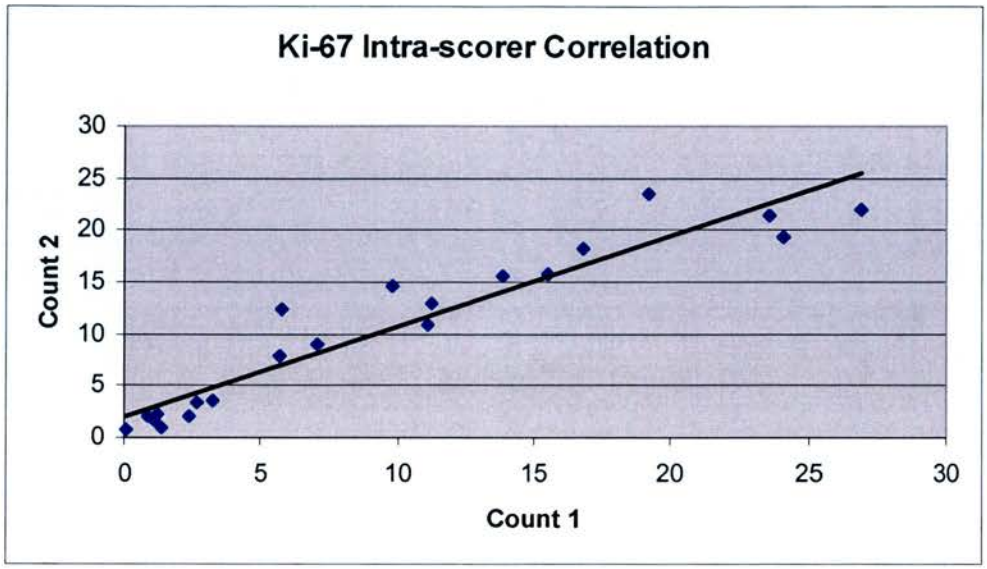

Figure 15: Intra-scorer correlation in scoring same slides. Pearson's correlation r = 0.95 (confidence intervals 0.88-0.98, p = <0.0001)

2.6.8 HER 2 (ErbB2) staining

To determine the HER2 status of tumours, HercepTest Immunohistochemistry was performed in an accredited laboratory. This was performed by the author in the Department of Academic Biochemistry in the Royal Marsden Hospital, London under the supervision of Professor M Dowsett. Control slides were used with every run. They were supplied with the HercepTest kit (see figure 16).

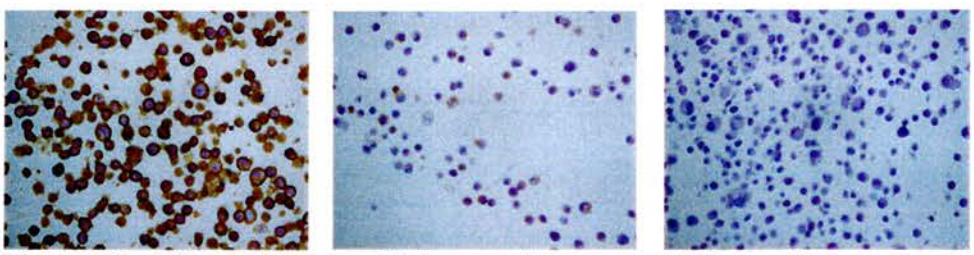

Figure 16: a) 3+ positive b) 1+ negative c) 0 negative
HercepTest control slides, see table 9

Paraffin embedded sections were dewaxed in xylene, then taken down through gradient alcohols (100% x3 then 90%, 80%, 70%) to distilled water. The slides were placed in antigen retrieval solution, then into a water bath heated to 97°C for 45 minutes and finally allowed to cool to room temperature. They were then washed with TBS. Sections were incubated in peroxidase blocking reagent for five minutes. They were then rinsed in distilled water and then in wash buffer.

Sections were then incubated in the primary antibody, rinsed in wash buffer and then incubated in the visualisation solution. They were again rinsed in buffer before DAB chromagen was applied to the sections. Following a further rinse in distilled water they

were counterstained with haematoxylin. Slides were then taken back up through gradient alcohol solutions to xylene before being cover plated. Full details of the technique are described later (see appendix 11).

2.6.9 Scoring of HER2

The intensity of specific membrane staining in the invasive cancer cells was assessed using the technique described in the training set that accompanies the materials (HercepTest kit scoring guidelines). The slides were viewed at x10 magnification under a light microscope. Staining was scored from 0 to 3 + as shown in table 9.

Staining pattern	Score	Her2 protein over-expression assessment
No staining is observed or membrane staining is observed in less than 10% of the tumour cells	0	Negative
A faint/ barely perceptible membrane staining is detected in more than 10% of the tumour cells. The cells are only stained in part of their membrane	1+	Negative
A weak to moderate complete membrane staining is observed in more than 10% of the tumour cells	2+	Weakly positive
A strong complete membrane staining is observed in more than 10% of the tumour cells	3+	Positive

Table 9: Cell membrane staining intensity criteria

Only membrane staining intensity and patterns were evaluated using the 0–3+ scale as illustrated by the HercepTest kit scoring guidelines above. Scores of 0 or 1+ were considered negative for HER-2 over-expression; scores of 2+ were considered weakly positive; and scores of 3+ were considered strongly positive. To qualify for 2+ and 3+ scoring, complete membrane staining of more than 10% of tumour cells had to be observed. Manual scores of 2 + were tested for amplification by fluorescence in situ

hybridization by staff in the Department of Academic Biochemistry in the Royal Marsden Hospital. The technique used is described later (see appendix 12).

To aid in the differentiation of 0, 1+, 2+ and 3+ staining, DAKO supplied an "Atlas for Interpretation of HercepTest™ Staining" and this was used. It shows representative pictures of the staining intensities (Figure 17).

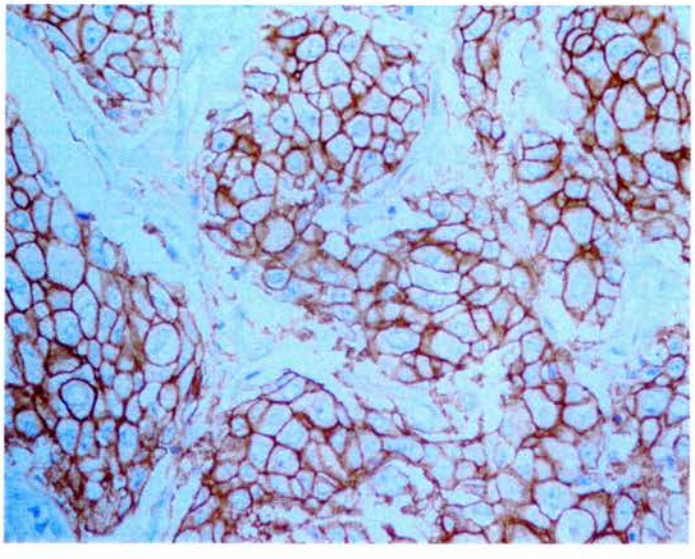

Figure 17: 3+ positive membrane staining for HER2

2.7 The Letrozole audit - study design

The audit was planned to investigate the use of letrozole as neoadjuvant therapy for a consecutive series of patients with large operable or locally advanced breast cancer. This was introduced as first line neoadjuvant treatment in 2001. Initially, a treatment period of three months of letrozole 2.5mg daily was planned. Patients were studied prospectively and data on response rates were collected. The primary endpoint of the audit was the response rate as measured by ultrasound and the rate of breast conserving surgery being performed in the group. Additionally, both fresh-frozen and fixed tissue samples were collected to look at the biological effects of the drugs.

2.7.1 Study population

137 post-menopausal patients with hormone receptor positive, large operable or locally advanced breast cancer were enrolled into this audit. All patients were treated in the Edinburgh Breast Unit at the Western General Hospital in Edinburgh between 14 May 2001 and 05 August 2004.

2.7.2 Inclusion criteria

- main breast cancer of any stage if loco-regional surgery was felt likely to be appropriate after a course of neoadjuvant treatment (T_{1-4}, N_{0-2}, M_{0-1})
- Tumour oestrogen receptor rich on core biopsy (Allred ER score 5-8). The vast majority of patients recruited were ER 7 or 8.
- Invasive breast cancer confirmed on core biopsy
- Postmenopausal (defined as one of the following;
    1. No spontaneous menses for at least 1 year in women >55 years
    2. Postmenopausal oestradiol levels (<5ng.dL)
    3. Bilateral oophrectomy
- Tumour able to be assessed by clinical examination, mammography and ultrasound scan
- A life expectancy of at least six months
- If large operable tumour, it needed to be >2cm on mammograms and ultrasound

Exclusion Criteria

- Premenopausal status
- Prior treatment with letrozole or tamoxifen
- Use of other investigational drugs within 30 days of starting letrozole
- Oestrogen receptor negative tumours
- Patients unable to give informed consent or unlikely to be compliant with treatment regimen

2.7.3 Trial protocol

1. Patients had a core biopsy performed at diagnosis. From larger tumours, extra cores were taken and stored fresh-frozen in liquid nitrogen to allow gene array work to be carried out. Patients completed a standard core biopsy consent form (See appendix 2) which gave permission to store fresh tissue taken at the initial diagnostic biopsy. This allowed the tissue to be collected for research purposes without the requirement for additional baseline core biopsies to be performed.

2. Postmenopausal patients subsequently identified at the staging meeting as having large operable or locally advanced oestrogen receptor rich cancer were invited to take part in the audit. They were given a patient information sheet (appendix 1). The option of neoadjuvant endocrine therapy and the alternatives were discussed with each patient and their relatives if present. 405 patients were screened and identified as potentially suitable. The majority of patients who discussed the option of neoadjuvant endocrine therapy agreed to take part in the audit.

3. Informed consent was not obtained from patients because letrozole already had a product licence for this indication (see appendix 13). Information about participation in the audit was sent to each patient's GP as part of their standard clinic letter.

4. Baseline tumour measurements were taken at the staging clinic once patients had agreed to take part in the audit. The diagnostic mammogram that had already been performed was used as the baseline. Clinical examination and ultrasound measurements were performed. Details of each patient's past medical history and current prescribed medication were documented.

5. Patients were initially prescribed three months of letrozole at a dose of 2.5mg daily.

6. Patients returned to clinic after 10-14 days of treatment. They were asked about any side effects or problems with the treatment. The visit included an optional 10-14 day core biopsy. It was explained to all patients that this biopsy would not influence their treatment and that the core biopsy was only being performed for research purposes. If they agreed to have this biopsy performed, they signed a consent form.

7. Patients returned again to clinic six weeks after the start of treatment. Again they were asked about any side effects of treatment. The tumour was measured clinically using callipers and by ultrasound scan. If there was any evidence that the tumour was not responding to letrozole and enlarging on treatment, a change of treatment was instigated as appropriate. If the tumour was responding a decision was made about surgery. All patients were assessed by a single consultant surgeon Mr Dixon, who operated on all these patients. He decided

whether the tumour was suitable for excision by breast conserving surgery, by mastectomy or whether a longer period of drug treatment was required. If surgery was planned, a date was organised.

8. Patients were seen in the breast clinic the day before their planned surgery. Repeat clinical examination, ultrasound scan and mammography were performed. Mr Dixon had a chance to review the patients and their response to neoadjuvant therapy and to make any final changes to the proposed surgery.

9. During surgery, fresh and fixed tissue from the cancer specimen were removed for analysis as previously described.

10. All patients who had responded to neoadjuvant letrozole continued on it for five years post operatively as adjuvant therapy. The audit patients were discussed in detail at both the pathology meeting and the multidisciplinary meeting following the surgical procedure. Further treatment was planned according to local protocols.

11. Patients continue to be followed up for disease recurrence and survival. Follow up data presented in this thesis was collected in May 2006 after a mean follow up period of 39 months (4-58).

2.7.4 Methods of collecting information

A proforma was completed for every patient. It collected data on the following;
date of entry to study, patient age, ER status, stage of tumour at diagnosis, type and date of surgery, start date of drug, any past medical history and any current medications.

Additionally, it was noted which investigator discussed the trial with the patient and which types of tissue sample were collected. Copies of any consent forms and pathology reports from the diagnostic core biopsy and final surgical excision specimen were filed within these proformas. Data were collected and filed at each clinic visit. Clinical measurements were recorded. Copies of ultrasound scans with electronic calliper measurements were filed. Mammographic measurements were also noted. These data were all collected prospectively which made data interpretation easier at a later date.

2.7.5 Laboratories used for tumour measurements

Tumour biological measurements were performed in the Edinburgh Breast Unit Research Laboratories in the Paderewski Building of the Western General Hospital, Edinburgh under the direction of Professor W R Miller and in the Royal Marsden Hospital, London under the direction of Professor M Dowsett. EGFR and HER2 assessment was also performed by Yuo Tao at Duke University, USA under the direction of Professor Matthew Ellis.

2.7.6 Letrozole audit summary diagram

New Patient Clinic	Potentially suitable patients identified. Core biopsy performed to confirm diagnosis, oestrogen receptor status and to obtain fresh tissue.
Day 0	Staging Meeting: Patient identified and audit discussed. Baseline clinical, mammographic and ultrasound scan measurements performed. Patients prescribed letrozole 2.5mg daily for three months (last dose to be taken on the day of surgery).
Day 10-14	Return to clinic. Any side effects documented. Optional core biopsy performed.
Week 6	Return to clinic. Any side effects documented. Ultrasound scan and clinical examination performed. Potential surgery discussed
Month 3	Return to clinic. Any side effects documented. Ultrasound scan and clinical examination performed. Mammograms performed. Surgery performed or continue on letrozole.

2.7.7 Study endpoints

- Characterising the clinical response to primary systemic endocrine therapy with letrozole in large operable and locally advanced breast cancer
- Comparison of the rate of breast conserving surgery after letrozole compared with the initial surgery that would have been required prior to neoadjuvant treatment.
- The effect of letrozole on ER, PgR and proliferation as assessed by Ki67. Other immunohistochemical analyses were also performed including erbB2 and EGFR. Further immunohistochemical analyses are being performed on these samples when information from the microarray analysis becomes available.
- Where fresh tissue is available, messenger RNA has been extracted from the primary tumours to allow gene array expression to be assessed using microarray techniques.
- Correlating biological results with clinical response to determine whether it is possible to identify markers of tumour phenotype that can be used to predict subsequent clinical response to treatment with 2.5mg letrozole for three months
- Determining the effects of treatment on these biological markers after 10-14 days and after 3 months and whether these changes relate to the clinical response seen over that period
- Identifying potential markers of resistance to endocrine therapy

2.7.8 Statistical analysis

Correlation coefficients were used to examine the relationship between the measurement methods and final pathological tumour size. However, since it is the magnitude of difference that is important, an alternative statistic of the sum of the square of the difference was calculated, along with the Mean Sum of Squares from the regression without a constant term. Significance tests were not performed.

Correlation coefficients were also used to examine inter-observer and intra-observer agreement on proliferation counts, while the kappa and weighted kappa were used to examine agreement in Allred proportion and intensity scores.

The Kaplan-Meier method was used to produce survival plots and mean times to failure.

Contingency tables were analysed with the Fisher's Exact Test and/or χ^2 test.

Ki67 values were not Normally distributed at later time points (14 days and three months), and were log transformed. Analysis of variance/ t-tests were used to analyse this data.

All significance tests were two-sided. Analysis was completed using Minitab and SAS.

Section 3: Results

3.1 Patient demographics

- All patients were recruited and treated in the Edinburgh Breast Unit at the Western General Hospital, Edinburgh.
- 137 patients were recruited to the audit between 18th May 2001 and 23rd September 2003.
- Nine patients had bilateral cancers. Both cancers were sampled and monitored in eight patients (35, 36, 46, 77, 78, 96, 104 and 127) and only one side was monitored in the final patient (10).
- Five patients had multifocal cancers within one breast. In these cases, each lesion within the breast was measured separately in three patients (5, 18 and 127) and the main lesion only was measured in two (75 and 112).
- 136 women and 1 man (patient 82) were included.
- The mean age of the study population was 76 at the time of diagnosis (range 55 to 93).
- Follow up data on patient survival were collected in May 2006 after a mean follow up period of 39 months (range 4-58 months).

3.2 Protocol violators

12 patients did not complete the three month treatment period for the following reasons;

- Five died before completing three months of treatment (1, 9, 54, 80, 113).
- One patient could not attend the breast clinic for follow up measurements because of poor health (37).
- One patient did not attend the breast unit for follow up after starting the audit and refused any further visits and treatment (98).
- One patient had disease progression at six weeks and proceeded to have chemotherapy instead (128).
- One patient was subsequently found to have a false positive ER value at diagnosis and the tumour was subsequently shown to be ER negative (86).
- Two patients stopped treatment after two weeks due to side effects thought to be related to starting letrozole (42 and 134).
- One patient elected to have surgery earlier than planned after only five weeks of treatment (patient 88).

3.3 Assessing clinical response

The detailed clinical response data (percentage reduction in tumour volumes on (i) clinical measurement, (ii) ultrasound scan and (iii) mammography over the three month treatment period) can be seen in Appendix 14. In general, there was agreement across the three modalities of tumour measurement about the degree of response to treatment. In each case where there was discrepancy between the results, the case was re-examined by all investigators. Where there was consensus, the patient was assigned to the most representative clinical response category. If no consensus was reached, the patient was excluded from the analysis.

Appendix 15 shows the comparison between ultrasound measurement and final pathological tumour size in the excised specimen of the first 50 patients. It shows the USS measurement before treatment, the USS measurement after treatment and the measured pathological size of the excised specimen. The pathological volume is likely to be an overestimate as only one tumour measurement (the largest) is recorded on the majority of pathology report forms and the volume calculated is therefore an estimate of the largest possible volume. Some tumours were not excised at the end of the treatment period so only cores were available.

Correlation coefficients were used to examine the relationship between the measurement methods and final pathological tumour size (Table 10). However, since it is the magnitude of difference between measurements that is important, an alternative statistic of the sum of the square of the difference was calculated (Table 10). Correlation alone was not used because, for example, if one measurement method always overestimated the final tumour size by a fixed amount, the correlation between methods of measurement would appear to be good but the measurement method would not be very accurate.

	Correlation with pathological size (p-value)	Sum of (method– final path)^2
Pre clinical	0.690 (<0.001)	108.24
Pre mammogram	0.703 (<0.001)	62.35
Pre ultrasound	0.642 (<0.001)	25.93
Post clinical	0.215 (0.22)	50.77
Post mammogram	0.311 (0.07)	27.35
Post ultrasound	0.230 (0.19)	23.37

Table 10: Correlation of non-invasive measurements with final pathological size

Comparing the volumes seen on clinical assessment, ultrasound measurement and mammographic measurement with final pathological size, it can be seen that tridimensional ultrasound was the most accurate way of measuring tumour response as it had the lowest deviance from the final pathological size.

3.3.1 Clinical response categories

On the basis of clinical response, patients were divided into the five response categories described earlier (on page 91). Figure 18 summarises the number of patients in each response category. 83 patients had a partial response, 25 had a minor response, 10 showed no change and six had disease which had progressed.

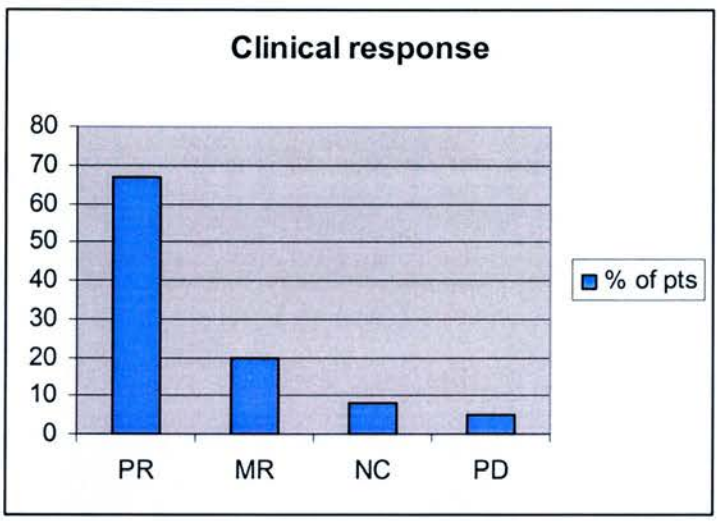

Figure 18: Clinical response to treatment (PR= partial response, MR= minor response, NC= no change, PD= progressive disease)

For the purpose of analysis, those patients achieving a greater than 50% reduction in tumour volume were categorised as responders (R). All other patients were categorised as non-responders (NR). Therefore 67% of cases were considered responders and 33% non-responders in this series.

3.3.2 Suitability for breast conservation

Of the 98 patients who went on to have surgery at the end of the treatment period, 41 patients were considered suitable for breast conservation at the outset of treatment (Figure 19 and Table 11).

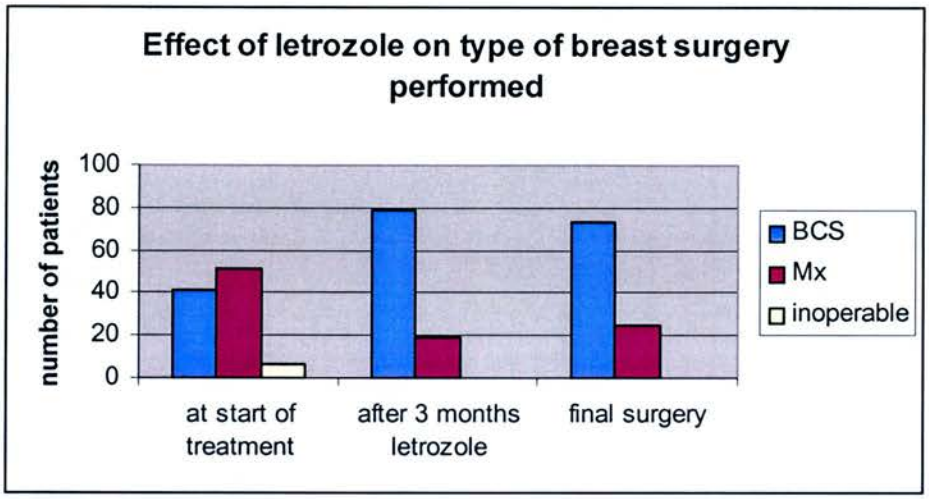

Figure 19: Effect of three months treatment with neoadjuvant letrozole on type of breast surgery performed. BCS = breast conserving surgery Mx = mastectomy

After the three month treatment period, 79 patients were able to have breast conserving surgery performed. However, 13 of these patients required a re-excision because of positive excision margins (16%). Of these, six patients had a mastectomy performed while another six had a re-excision of the cavity with clear margins. One patient who had involved margins was not considered fit to have a re-excision and therefore continued on letrozole with no clinically evident local recurrence to date.

	At time of diagnosis	After three months of neoadjuvant letrozole
No of patients suitable for breast conserving surgery	41	79
Number of patients requiring mastectomy	51	19
No with inoperable tumour	6	0
Patients meeting criteria for Axillary node sample*	10	61
Patients meeting criteria for axillary node clearance*	88	28
No axillary surgery done	N/A	9

Table 11: Effect of three months treatment with letrozole on type of surgery performed
*Edinburgh Breast Unit protocol is to perform axillary node sample for tumours under 2cm and axillary node clearance for tumours over 2cm or clinically involved nodes.

This means that 32 patients (63% of those patients who required mastectomy at the outset) had their surgical procedure downstaged from mastectomy to breast conserving surgery after three months of treatment with neoadjuvant letrozole.

Of the six initially inoperable tumours, four were ultimately able to have breast conserving surgery while two were downstaged to requiring mastectomy.

3.3.3 Axillary surgery

The majority of patients (90%) had large tumours prior to starting treatment and therefore would have had an axillary node clearance performed in combination with their breast surgery as part of Edinburgh Breast Unit protocols in place at that time. However, after treatment, 51 patients who would have been selected for an axillary clearance were considered suitable for axillary node sampling. In total, less than one third of patients (29%) had an axillary node clearance performed (Figure 20).

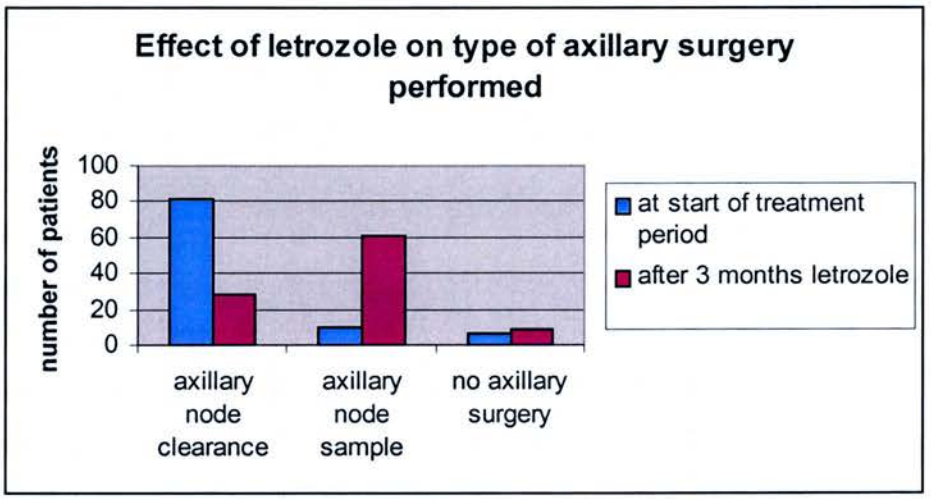

Figure 20: Axillary surgery planned prior to treatment and after three months treatment with neoadjuvant letrozole

There was a small group of patients (6%) at the start of treatment who were not suitable for surgery because their tumour was considered inoperable. Ultimately they all went on to have surgery. There were a small number of patients (9%) who did not have any axillary surgery performed along with their breast surgery. This was because they were frail and had excisional surgery performed under local anaesthetic.

3.3.4 Completeness of tumour excision and incidence of local recurrence

Only seven local recurrences (5%) have occurred in the series to date.

- Patient 20 had a local recurrence in her mastectomy wound nine months after initial surgery and subsequently died with evidence of distant metastases the following year with the cause of death being recorded as breast cancer.

- Patient 2 had a recurrence 13 months after her original wide local excision. This was treated with re-excision and radiotherapy. There had been a question about her original excision margins at the time of initial surgery. She died in January 2006 after 52 months of treatment with letrozole the cause of death being recorded as breast cancer.

- Patient 3 had suspected bony metastases at the time of her original diagnosis. This was later confirmed and she developed evidence of local recurrence in her wound 15 months after starting letrozole and one month before her death.

- Patient 11 developed local recurrence 22 months after starting letrozole and also developed bony metastases. She died eight months later from breast cancer.

- Patient 17 developed evidence of local recurrence after being on letrozole for 36 months. This was treated surgically and she remains alive and well.

- Patient 83 developed a local recurrence 39 months after starting letrozole and remains alive and well with no evidence of metastases.

- Patient 102 developed local recurrence which was also treated surgically and she too remains alive and well with no evidence of metastases.

Figure 21 shows the Kaplan-Meier curve for time to local recurrence. Mean time to failure (recurrence) = 38.03 months (se = 0.45) 95% CI (37.14, 38.92).

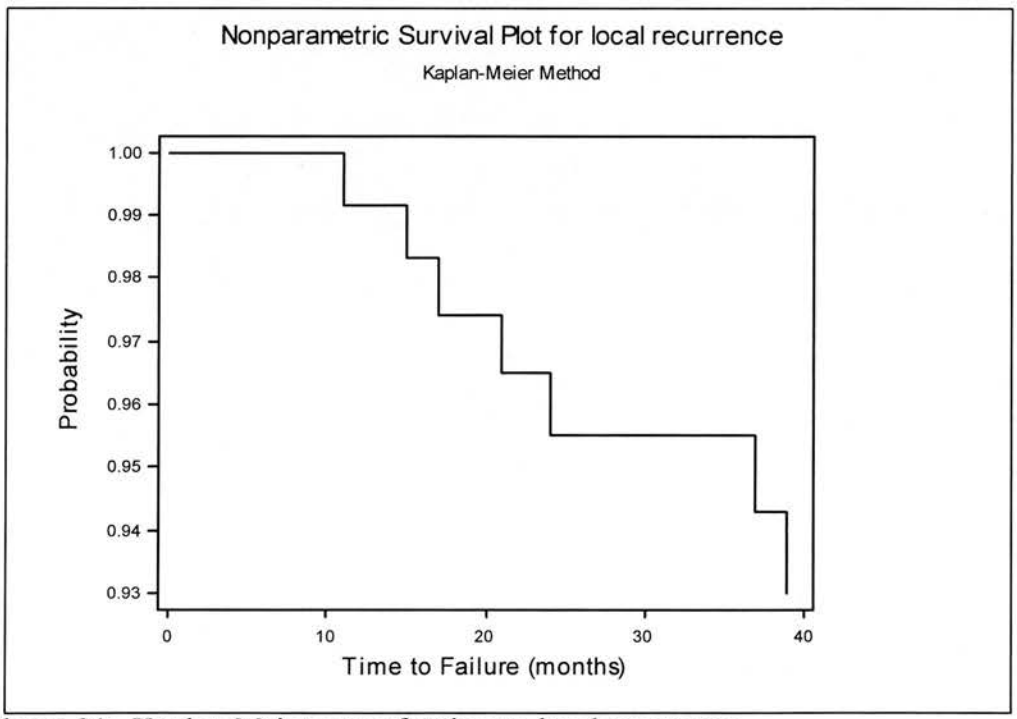

Figure 21: Kaplan-Meier curve for time to local recurrence.

3.3.5 Survival

Of the 125 patients who completed the three month audit period by May 2006 with a mean follow up period of 39 months (range 4-58), 42 patients had died. This is not surprising as the patients were elderly with significant comorbidity in addition to their advanced breast cancer at the time of initial diagnosis. Figure 22 shows the Kaplan-Meier survival curve for time to death. Mean time to failure (death) is 44 months (se = 1.42) 95% CI 41.2-46.8. It is not yet possible to calculate median survival time as the probability of death has not yet fallen below 50%. The lower quartile (25%) survival time is 31 months (ie the point at which 25% of patients had died, 75% survived).

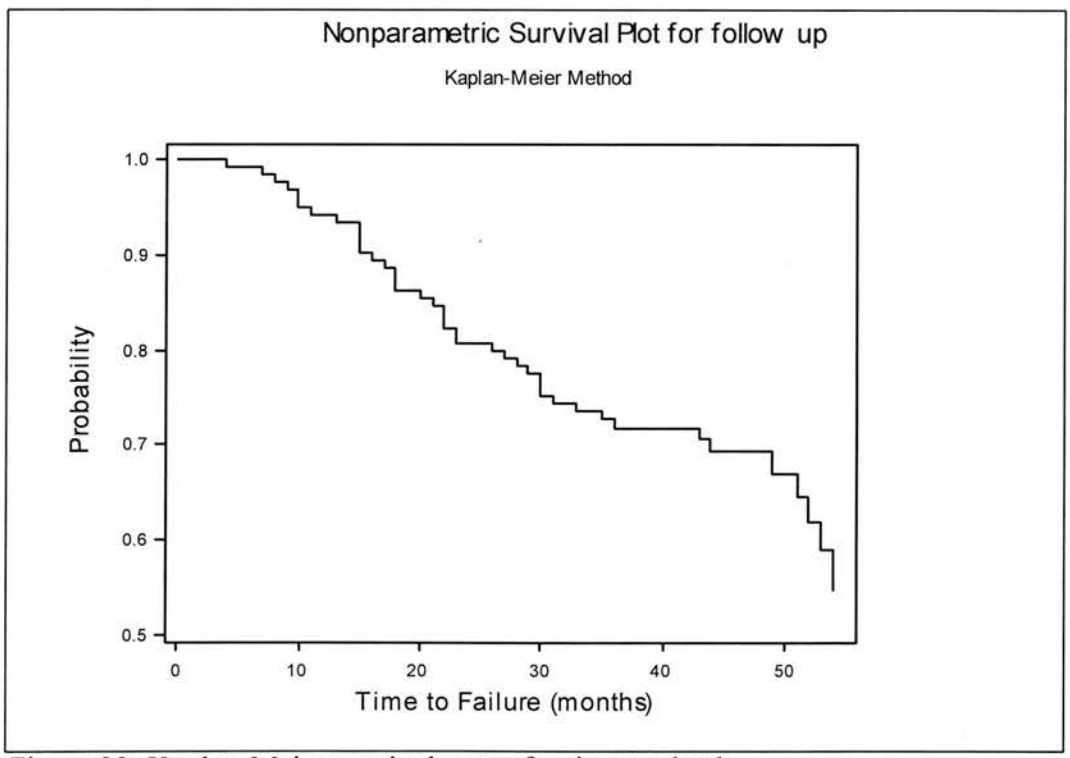

Figure 22: Kaplan-Meier survival curve for time to death.

Of the 42 patients who died, 23 had evidence of recurrent breast cancer at the time of death. Eighteen died of causes unrelated to their breast cancer. Figure 23 shows the Kaplan-Meier curve for time to breast cancer recurrence (local or metastatic) or death. Mean time to failure (cancer recurrence / death) is 39.9 months (se = 0.77) 95% CI 38.4-41.4.

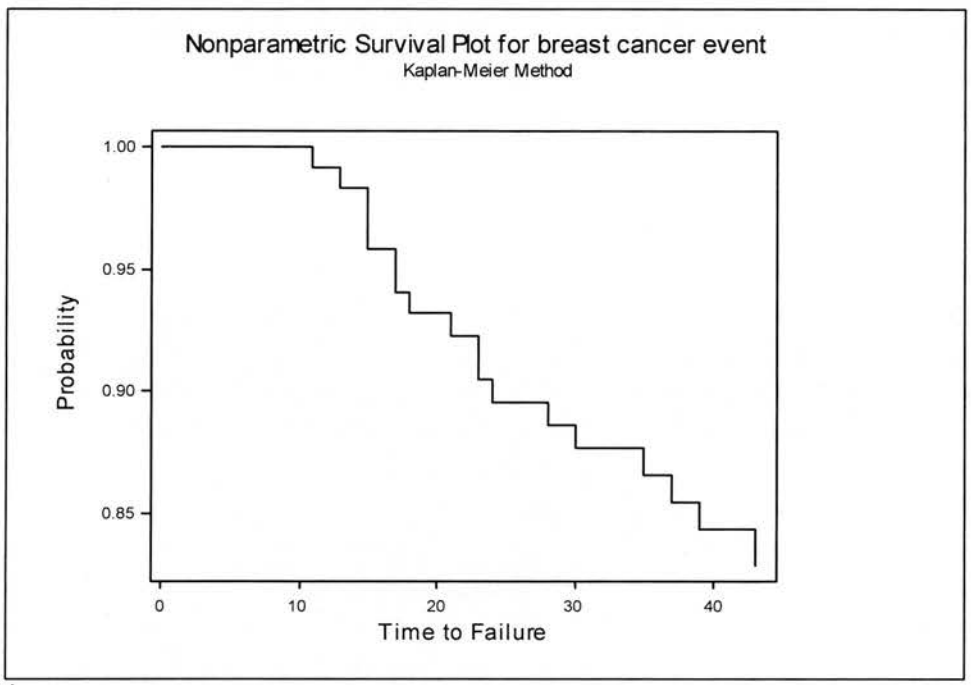

Figure 23: Kaplan-Meier survival curve for time to breast cancer recurrence or death.

83 patients remain alive, and 16 patients continue to take letrozole as the only treatment that they have had for their breast cancer with their disease under control.

3.4 Assessing biological markers of response

When determining the group used to compare clinical response with biological markers the following patients were excluded,

- Patients who did not have triple biopsies of fresh and frozen tissue at the time of diagnosis, at 10-14 days and at three months. They did not have enough cancer in the biopsies to allow all immunohistochemical staining to be performed and assessed.
- Any patient where there was doubt about the accuracy of the assessment of response eg invasive lobular carcinomas, mucinous carcinomas, multifocal tumours.

Since the 14 day tumour biopsy was optional, not all patients elected to have this performed. The final group analysed for markers therefore consisted of 62 patients who had a complete set of clinical and biological results available for comparison.

Appendix 14 shows a summary of the clinical, ultrasonic and mammographic responses to treatment for this group. Each of the 62 biological tissue study patients was assigned a response status on the basis of the modified WHO criteria across all three modalities and taking final pathological size into account (Appendix 16). Pathological response was also assessed according to the changes in histological features described on page 90. Each patient was also assigned to a pathological response category (See appendix 16).

3.5 Clinical response to letrozole

Overall, there were no complete responses (CR), 49 partial responses (PR), 11 minor responses (MR), 2 no change (NC) and no patient had progressive disease (PD) (Figure 24). For the purpose of analysis those patients achieving a greater than 50% reduction in tumour volume were categorised responders (R). All other patients were categorised as non-responders (NR).

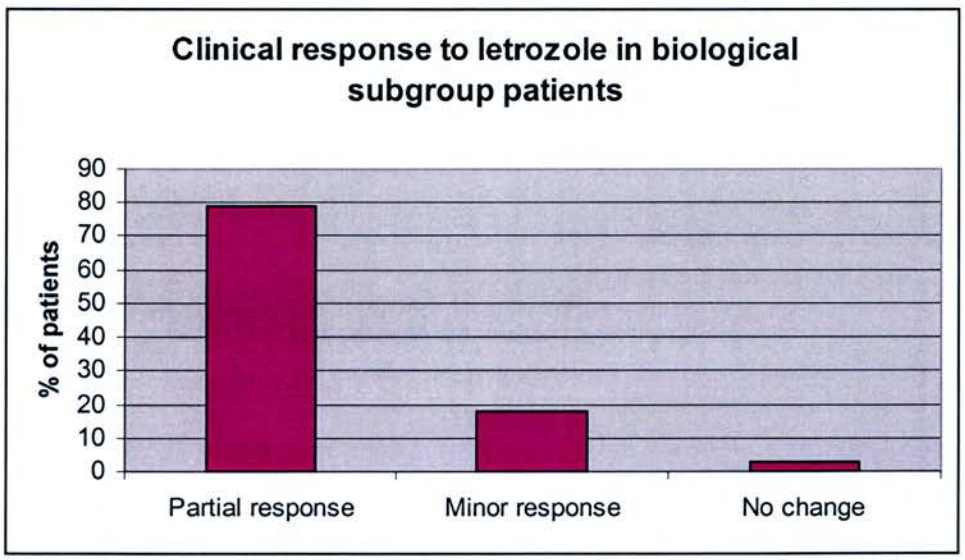

Figure 24: Clinical response to letrozole in biological subgroup of 62 patients

Using these criteria, 48 (77%) patients were considered clinical responders (>50% reduction in tumour volume at three months by serial ultrasound).

3.6 Histopathological assessments and changes

Marked changes in tumour morphology were seen after treatment with letrozole. Pathological changes were detected in more than two thirds of cases after drug treatment although these changes were not always consistent. In the majority of cases this comprised a decrease in cellularity and an increase in fibrosis but other patterns were also seen.

The following pathological features were specifically assessed in serial biopsies by the author together with a consultant pathologist (Prof T J Anderson),

- The proportion of cancer in the biopsy slides being assessed

- Cellularity, fibrosis, elastosis, lymphocytic infiltrate, necrosis, nuclear pleomorphism, the presence/absence of glands, mitosis.

These results can be seen in table 12.

Pathological feature	Change	% of cases changing
Cellularity	Increase	13
	Decrease	35
	No change	52
Fibrosis	Increase	44
	Decrease	30
	No change	26
Elastosis	Increase	35
	Decrease	30
	No change	35
Lymphocytic infiltrate	Increase	35
	Decrease	4
	No change	61
Necrosis	Increase	4
	Decrease	4
	No change	90
Nuclear pleomorphism	Increase	13
	Decrease	33
	No change	54
Number of glands	Increase	2
	Decrease	27
	No change	71
Mitosis	Increase	2
	Decrease	17
	No change	81

Table 12: Percentage of cases showing a change after three months of letrozole

Some features such as elastosis, showed no obvious pattern with a third of patients showing an increase, a third a decrease and the final third no change. More than a third of tumours showed an increase in lymphocyte infiltrate and only 4% had increased tumour necrosis. There were a small number of cases where there was almost complete pathological response to treatment with only microscopic foci of residual disease remaining. However, none of the patients in this series had a complete pathological response to three months of letrozole which might be seen more frequently after neoadjuvant chemotherapy.

The pathological changes were used to give each tumour a pathological response category similar to the clinical response category (See appendix 16). 46 (75%) of the 61 assessable tumours displayed evidence of a pathological response (Figure 22).

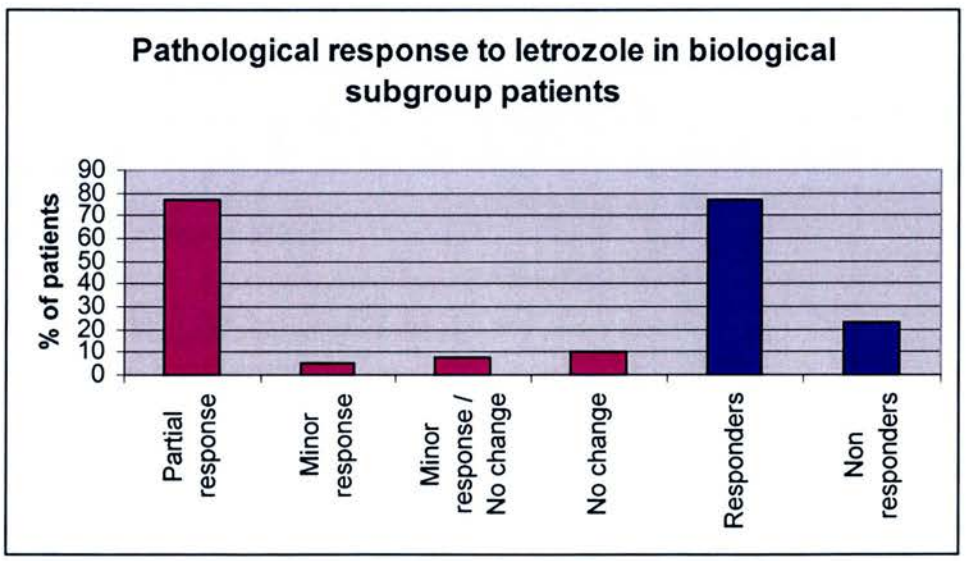

Figure 22: Pathological response categories after three months treatment with neoadjuvant letrozole

The decreases seen in nuclear pleomorphism and mitosis indicate that letrozole is capable of modulating cellular populations within individual tumours. These changes are generally towards a less aggressive phenotype. These changes are however minor when compared with the effects on Ki67.

There was a reduction in tumour grade seen after treatment which was usually a reflection of a reduction in mitotic index. A decrease in mitosis was seen in 17% of cases with only 2% increasing mitotic rate with treatment and the majority being unchanged.

3.7 Relationship between clinical and pathological response

The relationship between pathological response and ultrasound volume change can be seen in table 13. Overall looking at the whole group there was a significant correlation between changes in ultrasound volume and pathological changes. However, pathological changes were seen after treatment in some tumours that did not have a significant shrinkage in ultrasound volume (five cases) and conversely, there were a small number of tumours (seven cases) which did show a major shrinkage in ultrasound volume but did not have any evidence of pathological changes.

	Clinical response > 50%	Clinical response < 50%
Pathological response	41 (66%)	5 (8%)
No pathological response	7 (11%)	8 (13%)
Total	48 (77%)	13 (21%)

Table 13: The relationship between clinical and pathological response

Looking at the relationship between clinical and pathological response, one is seen to be a good predictor of the other (i.e. there is a lack of independence, $p= 0.0015$ by Fisher's exact test). In 80% of cases the responses agree.

Looking at the percentage reduction in tumour size on tridimensional ultrasound, the data are not Normally distributed and therefore require transformation for further analysis. The simple log transformation of fall = loge(101-reduction) converts the data to a more Normal distribution. Simple analysis of variance of the data reveal that a positive response from both the clinical and pathological tests are significant ($p<0.0001$ and 0.0002, respectively). The means and 95% CIs for the transformed data can be seen in tables 14 and 15:

	Mean response	95% CI
Clinical responder (n=48)	3.07	2.90-3.24
Clinical non-responder (n=13)	4.22	3.89-4.54

Table 14: Ultrasound response in relation to clinical response ($p<0.0001$)

	Mean response	95% CI
Pathological responder (n=46)	3.15	2.97-3.32
Pathological non-responder (n=15)	3.83	3.53-4.14

Table 15: Ultrasound response in relation to pathological response ($p=0.0002$)

3.8 Oestrogen receptor (ER)

All tumours in the study were ER rich (Allred score 6, 7 or 8) prior to starting treatment. It was suggested in the PO24 study [41] that patients with lower levels of ER do respond to letrozole but not to tamoxifen. However, the numbers in the low ER groups were small in that study. The policy in the Edinburgh Breast Unit is to only treat ER rich tumours with neoadjuvant endocrine therapy as those are the most likely to respond and to receive clinical benefit (high ER levels have a greater percentage reduction in tumour volume). Raw data for all ER scores in all biopsies can be seen later (Appendix 17).

In the biological subgroup of 62 patients that were studied in more detail, all tumours were either ER 7 (16 patients) or 8 (46 patients) (Figure 23).

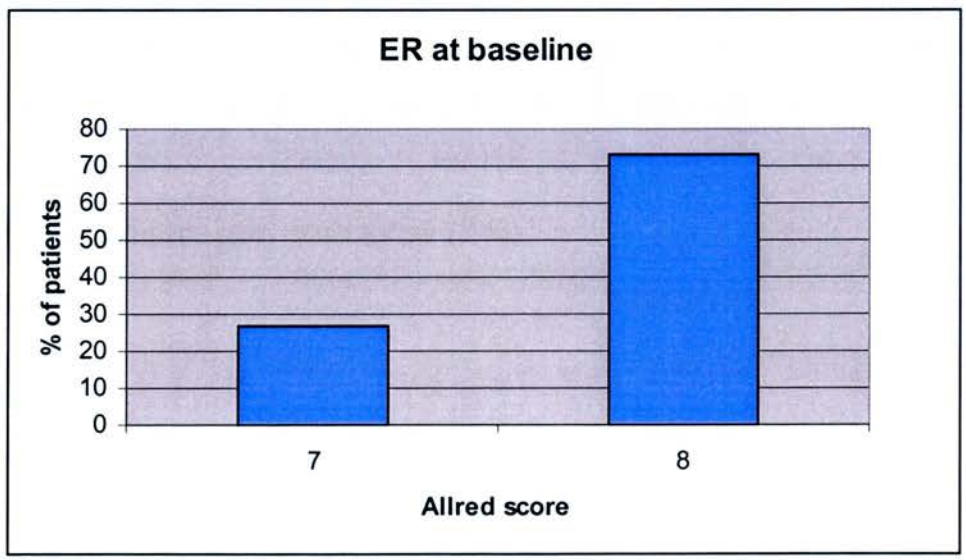

Figure 23: Baseline ER status

3.8.1 Effect of neoadjuvant letrozole on ER expression

The majority of cases (51%) showed no change in ER expression from baseline after three months treatment. Seven cases (11%) showed an increase of one Allred score from 7 to 8 with treatment (a change in intensity of staining). 24 cases showed a decrease in Allred score. In 21 of these this was a drop from ER 8 to 7, again a change only in the intensity of staining. The remaining three cases showed a drop of two Allred scores, from 5 to 4 for proportion and 3 to 2 for intensity. Table 16 shows the change in ER expression for the whole group.

Effect on oestrogen receptor of treatment with letrozole	Increase in Allred score	No change in Allred score	Decrease in Allred score
Number of patients	7	32	24
% of patients	11%	51%	38%

Table 16: Effect on ER expression of three months neoadjuvant treatment with letrozole

It is possible that the changes that were observed were partly due to the fact that the diagnostic and 10-14 day biopsies were core biopsies and the three month sample was cut from tissue blocks from the surgically excised sample in the majority of cases. The penetrance of fixative is better in core biopsies than in surgical specimens and this may be reflected in the decrease in intensity of staining in the surgical blocks which is reflected by a drop in Allred score. However, there were small changes in the ER score between the initial core biopsies and the 10-14 day core biopsies as well.

3.8.2 Effect of baseline ER on clinical response

Looking across the whole group, there were 83 patients whose baseline ER score could be correlated with clinical and ultrasound response accurately. This excludes protocol violators and patients with, for example multifocal disease or lobular carcinoma where the tumour measurements were felt to be unrepresentative. Of these, 60 had a baseline ER of 8, 3 were ER 6 and 20 were ER 7. For the purpose of comparison ER 6 and 7 were combined because of the small number of cases that were ER 6. Table 10 shows the response in these cases.

ALLRED ER score	No of patients	No. of responders	Median % reduction in tumour volume	
			Clinical	USS
8	60	48 (80%)	76*	67*
6 + 7	23	17 (74%)	63	48

Table 17: Response in 83 patients treated with three months of neoadjuvant letrozole subdivided according to ALLRED ER score. * $P < 0.05$

Response rates were similar in ER categories 8 and 6 +7 but there was a greater percentage reduction in tumour volume in patients whose tumours had the higher ER expression. This difference was significant ($p< 0.05$).

3.9 Progesterone receptor (PgR)

3.9.1 Baseline PgR expression

Of the 62 tumours, 56 (90%) were assessed as being PgR positive prior to treatment. Figure 24 shows the baseline PgR Allred score for the group and table 18 shows the percentage of patients in each group.

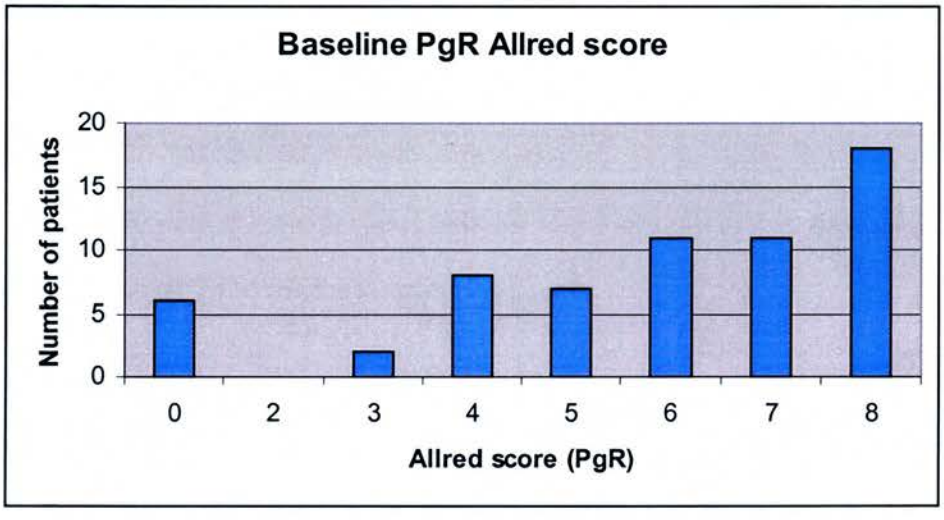

Figure 24: Baseline PgR expression categorized by Allred score

PgR Allred score	0	2	3	4	5	6	7	8
Number of patients	6	0	2	8	7	11	10	18
% of patients	10	0	3	13	11	17	16	29

Table 18: Percentage of patients in each Allred category score for PgR at baseline

3.9.2 Clinical and pathological response in relation to baseline PgR status

67% of the PgR positive tumours displayed a pathological response and 68% showed a clinical response (Figure 25). Of the six PgR negative tumours, four showed evidence of a pathological response and five showed evidence of a clinical response.

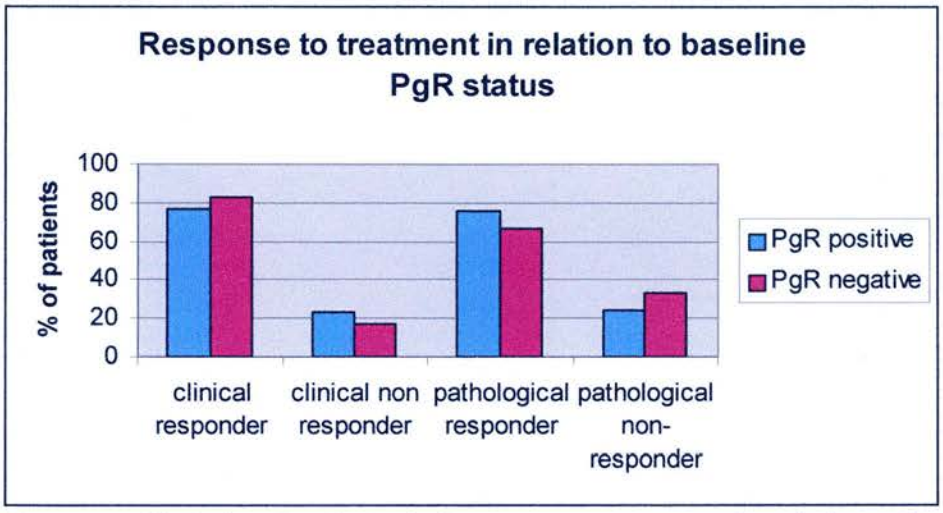

Figure 25: Clinical and pathological response to treatment in relation to baseline PgR

As can be seen the percentage of patients showing clinical and pathological responses were similar in both PgR positive and negative patients at baseline. Therefore, assessment of PgR adds nothing to ER in terms of selecting patients suitable for neoadjuvant endocrine therapy in this ER rich group. It may be that PgR positivity is more helpful in patients that have lower ER expression or are ER negative.

3.9.3 Effect of neoadjuvant letrozole on PgR expression

Treatment with letrozole caused a marked reduction in expression of PgR in 45 of the 55 receptor positive tumours (81%). In 38 cases (61%) this was a total loss of PgR expression and in 29 cases (47%) this occurred after only 14 days. This reduction in expression was irrespective of pathological or clinical response (see table 19 and figures 26 and 27).

	Receptor positive PgR lost (38 patients)	Receptor positive PgR decreased (7 patients)	Receptor positive No change PgR (10 patients)	Receptor negative No change PgR (7 patients)
Pathological response	25	7	9	5
No pathological response	12	0	1	2
Clinical response	30	6	7	5
No clinical response	8	1	3	2

Table 19: The relationship between change in PgR and clinical and pathological response

There is no evidence of a statistically significant difference in the response rates in the clinical responders vs non responders (p=0.85) or between the pathological responders vs non-responders (p=0.19). There was no evidence of a trend in either group.

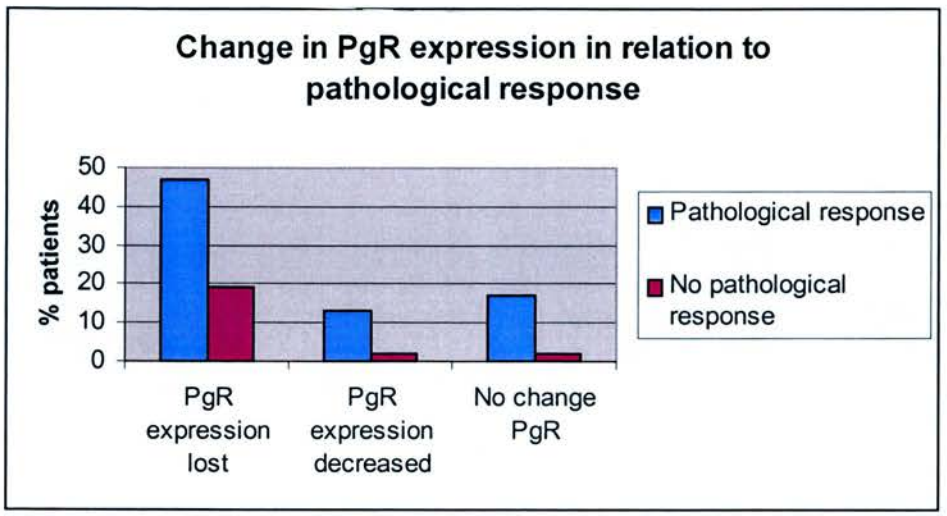

Figure 26: Change in PgR expression in relation to pathological response

Therefore it can be seen that the clinical utility of PgR as a predictor of response was limited. Some PgR positive tumours failed to respond to treatment while other tumours which were initially PgR negative responded to therapy. Responses were observed with all levels of initial PgR expression. Raw data for all PgR scores in all biopsies can be seen later (Appendix 18).

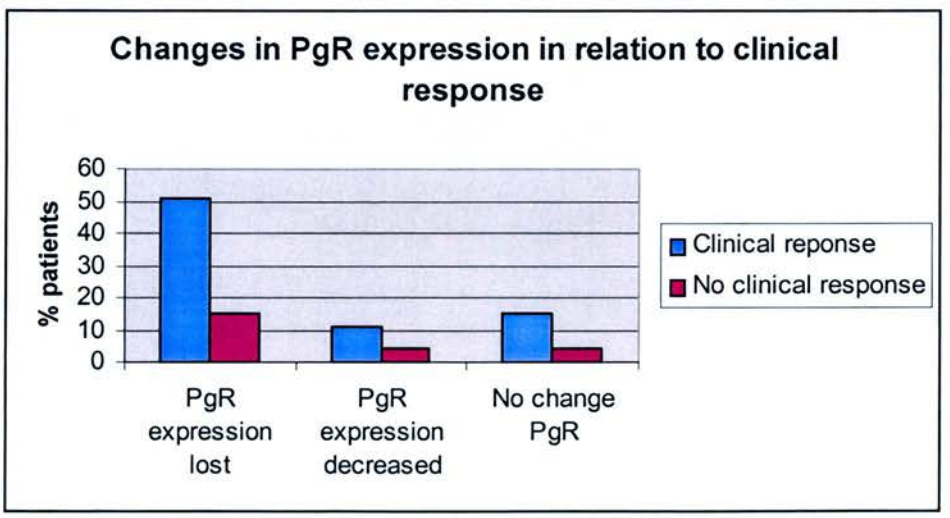

Figure 27: Changes in PgR expression in relation to clinical response

3.10 Effect on treatment on tumour proliferation

The raw data from the Ki67 assessment can be seen later (Appendices 19 and 20). A summary of the change in Ki67 expression in the biological subgroup of 62 patients with triple biopsies over the treatment period is shown in Figure 26. Measurement 1 was at baseline, 2 after 10-14 days and 3 after three months.

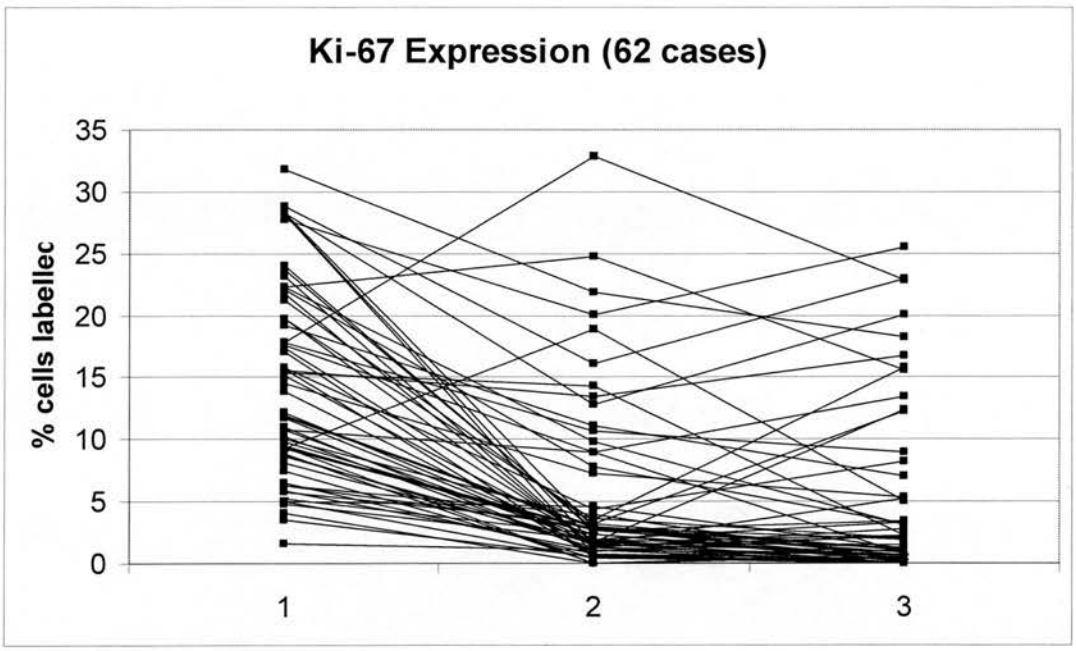

Figure 26: Summary of changes in tumour proliferation over three month treatment period with letrozole. 1= at baseline, 2= after 10-14 days, 3= after three months.

The data can be further broken down into the following subgroups;

- 22 patients in whom proliferation decreased at 10-14 days and then decreased further at three months (Figure 27).
- 26 patients in whom proliferation decreased at 10-14 days and remained down at three months (Figure 28).
- 11 patients in whom proliferation decreased at 10-14 days and then rose again at three months (Figure 29).
- Three patients in whom proliferation increased at 10-14 days and then decreased at three months (Figure 30).

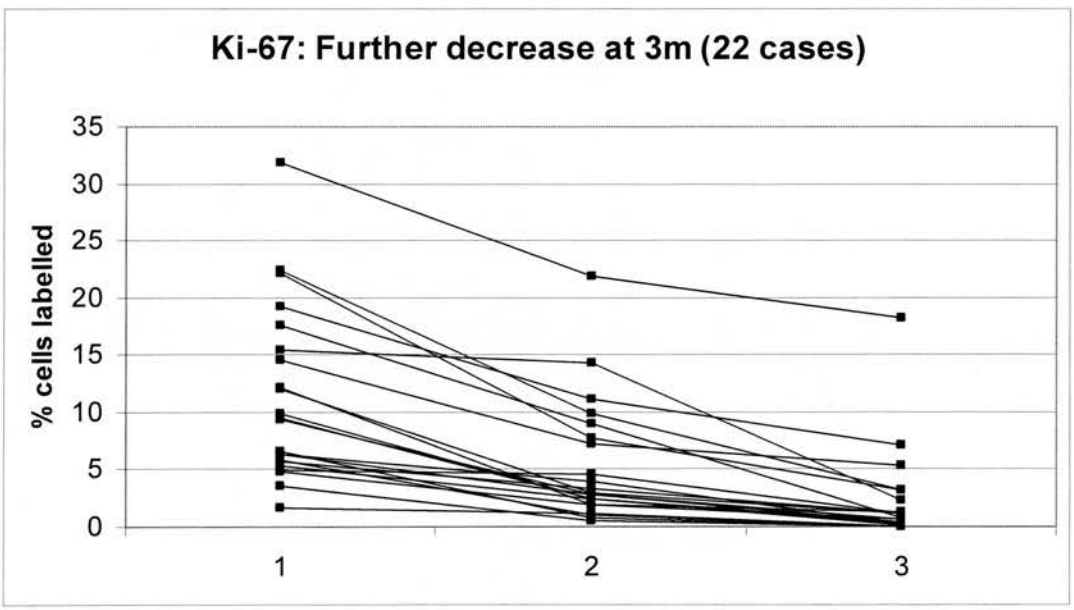

Figure 27: Patients in whom proliferation decreased at 10-14 days and then decreased further at three months. 1= at baseline, 2= after 10-14 days, 3= after three months.

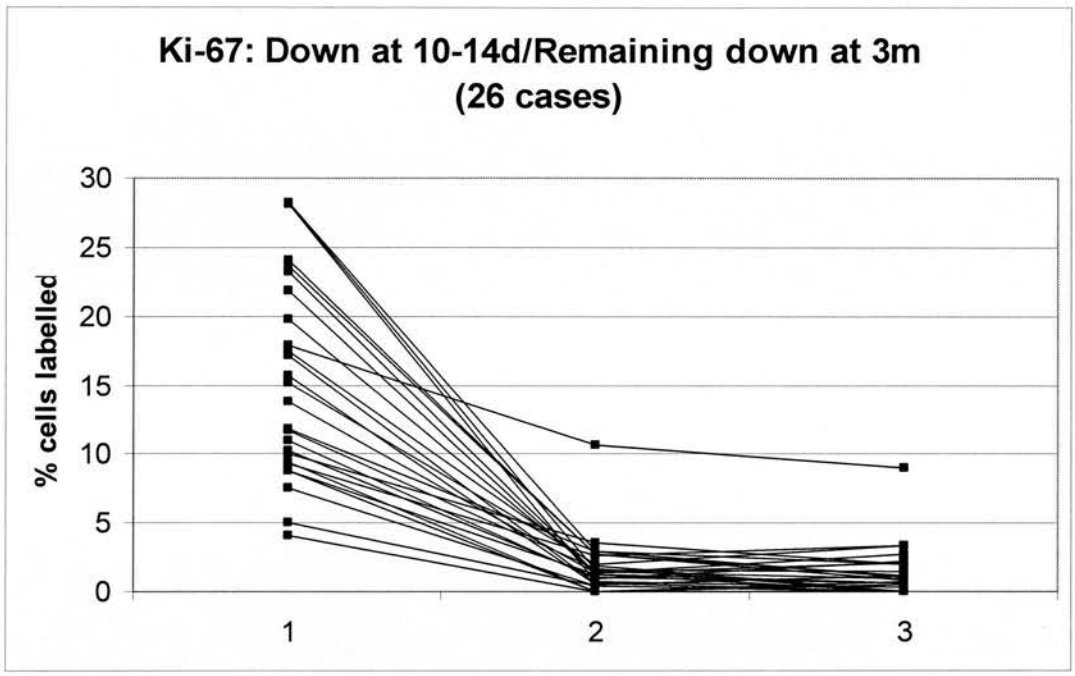

Figure 28: Patients in whom proliferation decreased at 10-14 days and then remained down at three months. 1= at baseline, 2= after 10-14 days, 3= after three months.

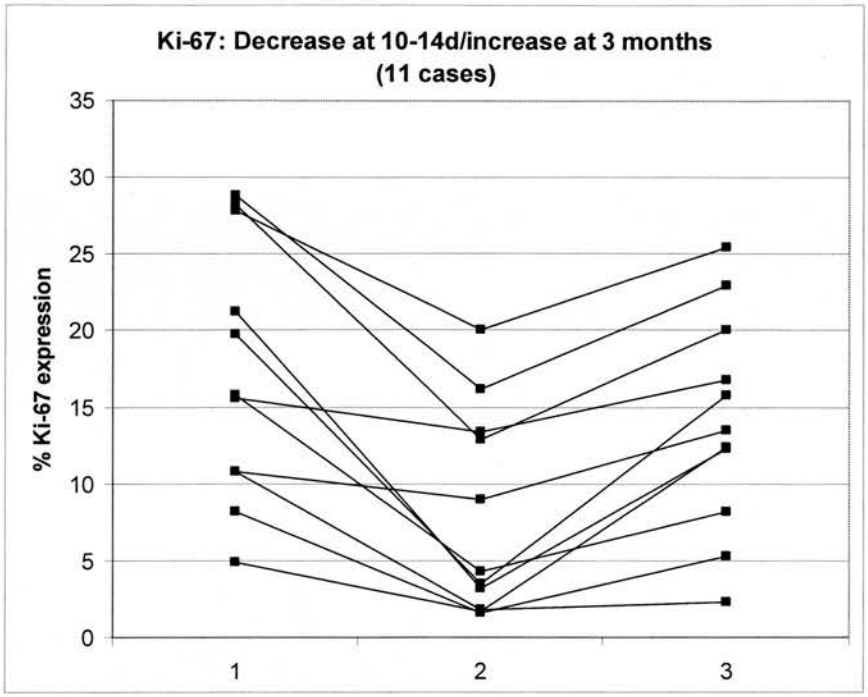

Figure 29: Patients in whom proliferation decreased at 10-14 days and then increased at 3 months. 1= at baseline, 2= after 10-14 days, 3= after three months.

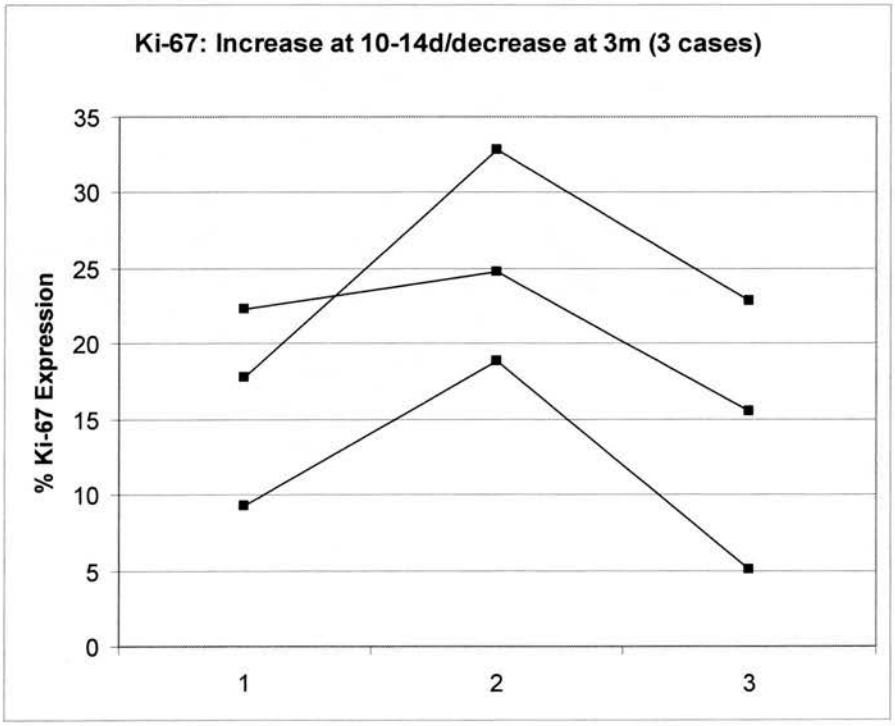

Figure 30: Patients in whom proliferation increased at 10-14 days and then decreased at three months. 1= at baseline, 2= after 10-14 days, 3= after three months.

Treatment with letrozole was associated with a marked decrease in expression of Ki67. 59 of the 62 patients showed a clear decrease in Ki67 staining after 10-14 days of therapy. This decrease was maintained or became greater in 48 cases; in the remaining cases proliferation increased again (in some cases to levels equal to or higher than their original proliferation). In the three cases that did not show a decrease at 10-14 days, although the levels did subsequently drop by three months they still remained similar to their initial pre-treatment values.

3.10.1 Correlating changes in proliferation with clinical response

Pre-treatment scores for Ki67 were similar in responders(R) and non-responders (NR). The mean initial proliferation in the responders was 14.04% (SEM 1.08). In the non-responders the mean initial proliferation was 15.79% (SEM 2.14).

3.10.2 Changes in proliferation at 14 days

Treatment was associated with highly significant decreases in all tumour sub-groups ($p< 0.005$ by paired Wilcoxon rank test) at 14 days (Table 20).

Levels in Ki67 14 days into treatment were not significantly different in clinical responders and non-responders ($p=0.34$), but scores were significantly higher in tumours which subsequently did not change morphologically compared with those showing a pathological response ($p=0.02$) (Table 20).

	Pre-treatment Mean (sem)	14 days Mean (sem)	? Significant fall
Clinical responders (n=49)	14.04 (1.08)	5.04 (0.96)	$P < 0.0001$
Clinical non-responders (n=14)	15.79 (2.14)	7.08 (2.15)	$P < 0.0001$
	P=0.46	P=0.34	
Pathological responders (n=47)	14.03 (1.09)	4.35 (0.89)	$P = 0.0002$
Pathological non-responders (n=15)	15.97 (2.22)	9.35 (2.22)	$P< 0.0001$
	P=0.40	P=0.02	

Table 20: change in proliferation compared to clinical and pathological response

3.10.3 Correlating changes in Ki67 at three months with clinical response

In the clinical responders group, 46 of the 49 patients showed a reduction in Ki67 from baseline at 3 months. Their mean drop in proliferation was 81.4% (SD 25.1). The three patients that showed an increase had a mean increase of 19% in proliferation (SD 10.3). Looking at the results for the whole group, there was a 75.23% decrease in proliferation (SD 34.5).

In the group of clinical non-responders, 12 out of 13 tumours showed a reduction in proliferation after three months of treatment. Their mean reduction in proliferation was 75.5% (SD 28.0). In one tumour the rate of proliferation increased by 43.1%. Taking the results for the whole group, the mean reduction in proliferation was 66.4% (SD 42.4). There was no significant difference in proliferation between the two groups ($p=0.43$, Student's two tailed T test).

The log transform $log_e(ki67+0.1)$ was used to Normalise the data prior to analysis (Table 21).

	3 months	Fall from pre-treatment
Clinical responders	0.21 (0.25)	2.27 (0.22)
Clinical non-responders	0.85 (0.46)	1.78 (0.40)
Significant difference?	P=0.22	P=0.28
Pathological responders	0.03 (0.24)	2.46 (0.21)
Pathological non-responders	1.40 (0.43)	1.22 (0.37)
Significant difference?	**P=0.0068**	**P=0.0047**

Table 21: Change in proliferation at 3 months

There is a statistically significant difference between pathological responders and non-responders in terms of the transformed ki67 value. This is evidenced in both the final 3 month value and in the difference between the pre-treatment value and the 3 month value. However, no significant difference was seen between clinical responders and non-responders.

3.10.3 Correlating changes in Ki67 at three months with cause specific survival

There was no significant difference in cause specific survival in relation to baseline Ki67 (p=0.6).

3.10.3.1 Proportional hazards analysis

To identify variables that may be associated with cause specific survival, the possible prognostic variables looked at were: year of treatment, age, size of tumour, grade, ER status, US response, baseline Ki67, % reduction in Ki67, operation and number of +ve nodes. Only 70 patients have data on all these variables. % reduction in Ki67 was the only one to show significance (p=0.01).

Removing operation and number of +ve nodes (as not all patients went on to surgery) allowed 84 patients to be included in the analysis. Again, only the reduction in Ki67 showed a significant correlation with cause specific survival (p=0.007).

If the percentage reduction in Ki67 was divided into two groups (<40% drop over three months and >40% drop) there was a significant difference in cause specific survival on univariate analysis (p=0.04). This can be seen in Figure 31.

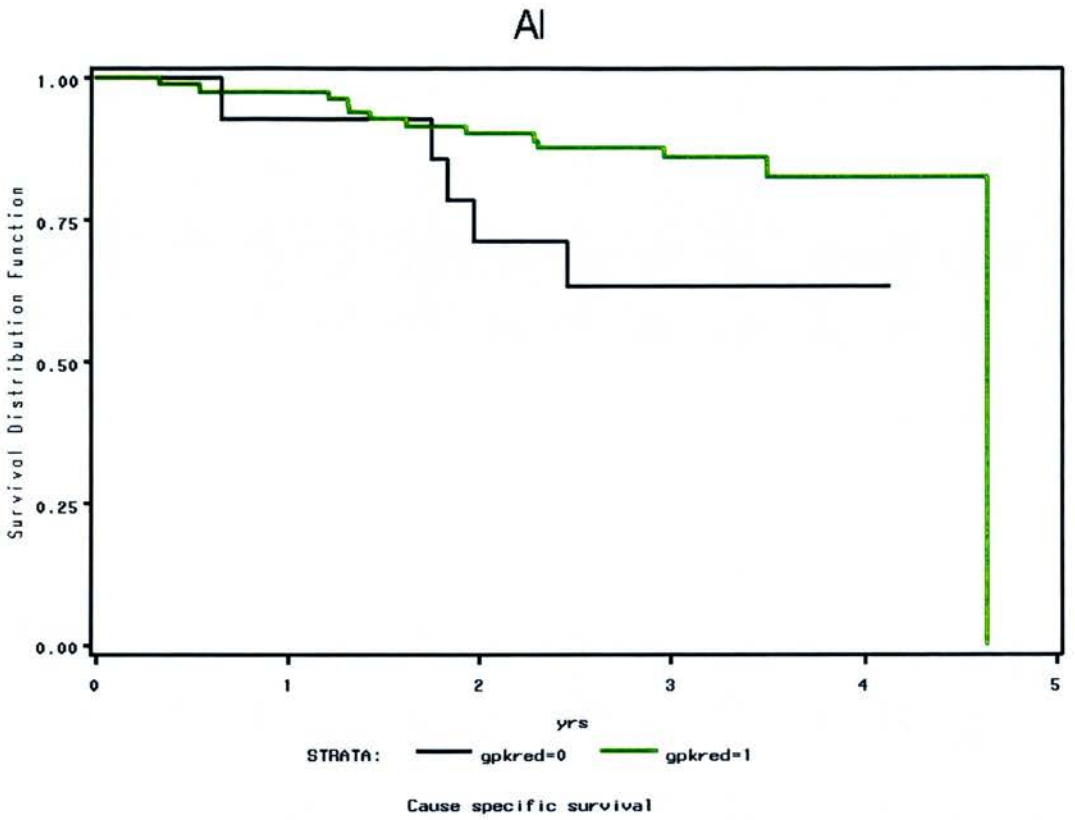

Figure 31: Cause specific survival in relation to drop in Ki67 (%) with treatment. Green line represents patients with >40% decrease in proliferation (83 patients) and black line represents patients with <40% decrease in proliferation (14 patients)

3.11 Optimum duration of treatment with letrozole

In this study, 42 patients were either deemed unfit for surgery, refused surgery, had responded but still required mastectomy or were still inoperable after three months treatment. They therefore continued letrozole for at least a further three months. 22 of the 42 were still taking letrozole at 12 months. It is useful to look at this group separately to assess whether these unselected tumours continue to respond to neoadjuvant letrozole for periods longer than three to four months. The following reductions in clinical tumour volume were calculated:-

% reduction in first three months, (volume at three months/volume at diagnosis x 100%)

% reduction between three to six months (volume at six months/volume at three months x 100%)

% reduction between 6-12 months (volume at 12 months/volume at six months x 100%).

Median % reduction in the tumour volumes from 0 - 3 months, from 3-6 months and from 6 -12 months are shown in table 22.

	Number of Patients	Median % reduction	95% CI
% reduction from 0 - 3 months	42	52	37-62
% reduction from 3 - 6 months	42	57	26-100
% reduction from 6 - 12 months	22	66	22-100

Table 22: Median percentage reduction in tumour volumes over treatment period

Tumours continued to reduce in volume during the 12 months study period. At three months there were 4/42 (9.5%) complete responses, by six months there were 12 / 42 (29%) and by 12 months 8 / 22 (36%). Only one patient who was responding at three months had disease progression at 12 months.

Neoadjuvant letrozole therefore produces ongoing tumour shrinkage in postmenopausal women over 12 months in these large operable or locally advanced ER + breast cancers. Patients whose tumours are responding to letrozole at three months can expect further reduction in tumour volume with continued treatment.

3.12 The effect of treatment on HER 2 (erbB2) status

The HER2 status of all tumours was checked on all three consecutive biopsies. There was no change in the expression of HER2 during treatment with letrozole. Those tumours which were positive on initial diagnostic biopsy were positive on all subsequent biopsies. Those that were negative remained negative after treatment with letrozole. All diagnostic biopsies had FISH performed by Yu Tao and Matt Ellis in Duke University to confirm the immunohistochemical findings.

3.12.1 Clinical response in HER 2 positive cancers

There were 12 cases (19%) in the biological subgroup of 62 patients which over-expressed HER 2 (Table 23). This is a slightly higher percentage than might be expected for an ER rich group (~10%) but the tumours were all locally advanced at presentation so perhaps more aggressive which might account for the higher than expected rate of over-expression of HER 2. The average age of the HER 2 +ve group was 73 compared with an average age of 76 for the whole group.

Audit number	Patient age	Path Resp	Clin Resp	Survival
20	55	PR	PR	Died
35	78	PR	PR	
51	55	PR	PR	
52	79	PR	NC/MR	Died
57	76	PR	NC	
84	82	MR	NC	
94	80	PR	PR	
95	67	NC	NC	
109	79	MR	NC	
120	65	PR	PR	
125	86	PR	NC/MR	
136	77	MR	PR	

Table 23: Patients over-expressing HER2

The response rate is shown in Table 24. The response rate was lower in the group that over-expressed HER2 than the negative group but this was not significant. Therefore in this series HER2 status does not aid prediction of clinical response to letrozole.

HER 2 expression	Clinical Responder	Clinical Non-responder	Pathological responder	Pathological Non-responder
Positive	8 (67%)	4 (33%)	6 (50%)	6 (50%)
Negative	42 (82%)	9 (18%)	41 (82%)	9 (18%)

Table 24: HER 2 expression in relation to clinical and pathological response

Section 4: Discussion

The aims of this thesis were,

- to further characterise the clinical response to primary systemic endocrine therapy in breast cancer in the setting of a clinical trial
- to determine whether it is possible to identify biological markers of tumour phenotype that can be used to predict subsequent clinical response to neoadjuvant treatment with 2.5mg letrozole for three months;
- to determine the effects of treatment on biological markers after 10-14 days and after three months and investigate whether these changes correlated with clinical responses seen over that period; and
- to identify potential markers of resistance to endocrine therapy.

The discussion will first review the current status of neoadjuvant endocrine therapy and the role of letrozole in this setting. It will compare and contrast the findings of this study with other similar work in this field. It will discuss various methods of assessing clinical response and whether biological markers and short term clinical response might predict for longer term disease recurrence and disease free survival. Biological responses to neoadjuvant endocrine therapy after 10-14 days and three months treatment will be described as will the optimal time of drug treatment prior to surgery. The conclusions will summarise the study findings and suggest potential areas for future work.

4.1 Neoadjuvant endocrine therapy

Neoadjuvant therapy to treat breast cancer has to date predominantly consisted of chemotherapy. Neoadjuvant treatment with tamoxifen has been used in the past but it has been shown to have poorer outcomes in terms of disease recurrence and survival than when used in combination with surgery [34]. Neoadjuvant chemotherapy is often poorly tolerated in elderly patients where the morbidity of treatment and their co-morbidity are significant factors. This age group currently comprises a third of all breast cancer so it is important to find well tolerated effective treatment for them.

Clinical response rates can be compared with neoadjuvant chemotherapy regimes, which generally have a response rate of around 80% regardless of the regime used [140]. A Russian group have presented a study suggesting that there were similar clinical outcomes following neoadjuvant endocrine therapy or chemotherapy [115]. In their study, 121 postmenopausal women with ER +/- PgR positive breast cancer were randomized to receive neoadjuvant chemotherapy (four cycles every three weeks of doxorubicin and paclitaxel) or neoadjuvant endocrine therapy with either anastrozole or exemestane for three months prior to surgery. All baseline characteristics were similar in the groups apart from a slightly higher number of elderly patients (aged over 70) in the endocrine treated group (32% compared with 20%). Both clinical and mammographic response rates were similar for both groups with a trend towards increasing breast conservation surgery in the endocrine treated group. There was no significant difference in local

recurrence rates after a mean follow up period of 34 months. However, more toxicity was reported in the chemotherapy group with significant numbers of women reporting alopecia (79%), neutropenia (43%), neuropathy (30%) and cardiotoxicity (7%). Side effects that were reported with endocrine therapy included hot flushes (23%), fatigue (15%), vaginal bleeding (7%) and arthralgia (7%). Despite the small numbers, these results were encouraging in suggesting that neoadjuvant endocrine therapy has a similar response rate to chemotherapy with less associated toxicity. This may be particularly important in elderly patients who are less likely to tolerate chemotherapy.

In the current study, neoadjuvant treatment with letrozole has been shown to be effective in the population group studied with 67% of patients responding positively to treatment. This was an elderly group with a mean age of 76 yet treatment was well tolerated with only two patients stopping treatment because of side effects. 32 patients (63%) were able to have their surgery down-staged from mastectomy to breast conserving surgery, another factor in this population since mastectomy has a 1% associated mortality in this age group.

Response rates to treatment appear better than those seen previously with neoadjuvant tamoxifen. The response rate of 67% in this study is similar to the response rate of 60% seen in the PO24 study [41] with neoadjuvant letrozole compared to a 41% response rate for tamoxifen in that study and in earlier studies [38]. This study selected patients whose tumours were ER rich which may partially explain the higher response rate.

Differences in response to letrozole compared with tamoxifen may be related to increased efficacy. Letrozole causes oestrogen levels to be suppressed almost immediately whereas tamoxifen takes weeks to reach steady-state levels. [141]

Letrozole also appears to have fewer side effects than tamoxifen and is better tolerated [142]. It does not have the increased rate of endometrial cancer or thrombo-embolic events that are associated with tamoxifen [142]. However, there are concerns about the potential long term effects of the profound drop in oestrogen caused by aromatase inhibitors and their effect on bone, lipid and coagulation markers. Studies are ongoing to further investigate these potential side effects and morbidities.

4.2 Assessing clinical response to primary endocrine therapy

Neoadjuvant therapy allows direct assessment of tumour sensitivity to endocrine therapy by monitoring tumour response in situ. There are several methods of assessing response including clinical examination by measuring with callipers, mammographic measurements and serial ultrasound scans. Ultrasound scanning has been previously shown to correlate most closely with pathological size in the excision specimen [135]. However, ultrasound scanning is operator dependent so it was important to check its accuracy. In this study, ultrasound scanning was the modality that corresponded most closely with pathological size.

In the majority of cases, there was satisfactory correlation between all modalities used to measure a tumour. However, some patients' tumours presented a challenge. Multifocal tumours were difficult to assess, especially once they had shrunk considerably. Invasive lobular carcinomas were difficult to measure accurately either clinically or using imaging. In some patients, it was impossible to assess the tumour using all three modalities at all time points. For example, in very large ulcerating tumours an accurate ultrasound measurement was not possible. Additionally, some patients were not able to tolerate mammography.

Accurate assessment of response was particularly important when comparing clinical response with corresponding biological responses. For this reason, patients were

excluded from the final series where it proved difficult to assess clinical response accurately.

It was also important to assess tumour response using pathological determinants in addition to the more traditional clinical tumour measurements. This is because some tumours may have responded, not by getting smaller but by reducing cellularity and becoming fibrotic.

There was correlation between clinical and pathological response in the majority (84%) of cases. However, pathological changes were seen after treatment in some tumours that did not have a significant shrinkage in ultrasound volume (four cases) and conversely, there were a small number of tumours (six cases) which did show a major shrinkage in ultrasound volume but did not have any evidence of pathological changes. This could be explained if tumours shrank without altering their microscopic structure. A similar effect has been described in patients being treated with neoadjuvant chemotherapy.

When looking at endpoints such as proliferation, it was important to look at both clinical and pathological responses separately since, in some cases, there was a significant correlation with one but not the other. For example, tumours in which there was a pathological response had a significant drop in proliferation compared to pathological non-responders but this was not the case when looking at clinical response categories.

4.3 Clinical response to neoadjuvant letrozole

There have been few published studies examining the use of letrozole as neoadjuvant therapy in breast cancer. Small pilot studies in Edinburgh showed letrozole to be effective and well tolerated in this setting. The patients selected were postmenopausal and had large operable or locally advanced ER positive breast cancer. In one of the earliest studies, 24 postmenopausal patients were treated with 3 months of letrozole prior to surgery. A clinical response rate of 92% was seen and 15 of the patients who required mastectomy preoperatively were able to have breast conserving surgery after neoadjuvant treatment [101]. The PO24 study [41] is the only other large neoadjuvant trial using letrozole which has reported its findings. The study design and findings are discussed in detail on pages 54 and 55.

67% of tumours in this current study responded to treatment with neoadjuvant letrozole when assessed by ultrasound compared to a 35% clinical response when assessed by ultrasound in the PO24 study (see table 4). It is important, however, when comparing results of different studies to look at the differences between the methods used in clinical assessment.

In the PO24 study, the clinical response rate varied from 55% when using calliper measurement to 34% when using mammography and 35% when using ultrasound. Calliper measurements have been shown to be operator dependent and not to correlate as

accurately with final pathological size as ultrasound measurements [135]. In the PO24 study, the calliper measurements were performed by many different individuals in different centres and are therefore even less likely to be accurate and reproducible. There was no mention of comparison with final pathological tumour size or why calliper assessment was chosen as the primary endpoint. Additionally, the US measurements in the PO24 study were also performed in several institutions by many different clinicians. In the study described in this thesis, all measurements were performed in one centre by one of two trained clinicians using a single machine and probe.

It remains to be seen whether the degree of short term response predicts for overall disease free survival in this study group as the follow up period is currently still relatively short (mean follow up period of 39 months). Indeed, early promising results from treatment with neoadjuvant tamoxifen have produced disappointing long term results. The most recent update on a large study comparing tamoxifen treatment alone with surgery plus tamoxifen, shows that although most tumours initially respond, local disease control is poor when treated with endocrine therapy alone and survival from breast cancer is poorer in the tamoxifen alone group [40].

For that reason, the majority of patients in this study had surgery after three months. Many patients would have been quite happy to continue on letrozole when their tumour was responding and thus avoid surgery. However, it was felt that, for those patients who were fit enough to undergo an operation after three months treatment, this would be the optimal treatment. Only patients who refused surgery, were not fit enough for surgery

or still had inoperable cancers continued on letrozole for longer than the three month study period.

From the tamoxifen trials, it would appear that most patients will derive benefit from having surgery and that the majority will eventually acquire resistance and require surgery at some stage. However, the fact that patients can be treated successfully with neoadjuvant letrozole for longer periods may relate to the improved efficacy in comparison with tamoxifen. This means that some patients may be treated for long periods with letrozole with no adverse effect on their local disease control and survival. Additionally, more patients may be able to have their surgery downstaged from mastectomy to breast conserving surgery which is important in an elderly population with significant co-morbidity. In this study, 58% of patients treated with letrozole went on to have breast conserving surgery performed (74% of those undergoing any type of surgery). This compares with 48% of patients receiving letrozole in the PO24 study who underwent BCS and 36% of those treated with tamoxifen. BCS has a lower attached morbidity and mortality and if necessary can be performed under local anaesthetic.

4.4 Biological response to neoadjuvant endocrine therapy

Several studies have linked the rate of tumour proliferation to the response seen with neoadjuvant therapy [143-145]. Tumours with high rates of proliferation tend to have a good response to neoadjuvant chemotherapy [146]. They have also been shown to respond to neoadjuvant endocrine therapy. It has also been shown that successful neoadjuvant treatment is accompanied by a decrease in tumour proliferation [147]. To date, it has not been demonstrated whether early changes in proliferation predict for subsequent clinical response.

Tumour cell proliferation was assessed in this study to allow the use of proliferation as both a predictor and reflector of tumour response to be analysed. Ki67 has been shown to be an accurate and reproducible way of assessing proliferation in breast cancers. It can be assessed using immunohistochemistry on paraffin embedded slides. This study has demonstrated that tumours which respond to neoadjuvant endocrine therapy have a wide range of initial proliferation values. The proliferation has been shown to drop significantly as early into treatment as 10-14 days after treatment with letrozole.

Looking at the results in terms of a group of responders and non-responders, proliferation drops more in the tumours which show a clinical response. However, this cannot be applied on an individual basis to predict whether or not a tumour will respond to treatment.

4.5 Oestrogen receptors

All tumours in this study were ER rich with an Allred score greater than 6. Even within this select group of ER rich tumours, response to endocrine therapy was shown to vary with level of ER expression. Response rates were similar in ER categories 8 and 6 +7 but there was a significantly greater percentage reduction in tumour volume in patients whose tumours had the higher ER expression ($p< 0.05$).

When looking at clinical response in patients with lower ER scores (patients with ER 3-5) in the PO24 study [41] there was response to letrozole but not to tamoxifen. However, the numbers were very small so the authors acknowledged that this would require confirmation. Both agents showed a decreasing likelihood of response with decreasing ER score. For both drugs, the relationship between ER expression and log odds of response fitted a linear model that was significant by logistic regression within treatment groups (letrozole $P=0.0013$ and tamoxifen $p=0.0061$). Letrozole response rates were numerically superior to tamoxifen in every Allred category from 3 to 8 but this was not statistically significant.

This finding suggests that only patients whose tumours expressed a very high level of ER (Allred score 6 or above) are likely to derive benefit from neoadjuvant treatment with endocrine drugs. This is in keeping with the results of the study reported here where a higher response rate was seen in tumours with higher ER expression. Further

work needs to be performed to ascertain the role of neoadjuvant aromatase inhibitors in tumours that express lower levels of ER.

It is very important in the elderly population, who are most likely to be treated with neoadjuvant endocrine therapy, that there is a high likelihood of response to treatment. A delay in surgery should be avoided in this population. Therefore, baseline ER expression can be used to accurately target the group most likely to derive benefit from this treatment.

The lack of a consistent effect on ER expression seen in this study is similar to that reported in other studies using the aromatase inhibitors[148]. It is also the same as was reported in the PO24 study. However, it is different from the response that has been seen with tamoxifen which tends to reduce ER expression [149].

4.6 Progesterone receptor

PgR is regulated by oestrogen and is also a marker for ER mediated transcription.

The progesterone receptor can be regarded as a marker of oestrogenic activity as the protein is expressed as a result of oestrogen signaling which is mediated by a functional oestrogen receptor. For this reason, it has been suggested that PgR may be useful as a predictive parameter for response to endocrine treatment.

Change in PgR expression can also be used as evidence of the anti-oestrogenic mechanism by which aromatase inhibitors work. In this study, 47 of 57 PgR positive tumours showed a reduction in staining after treatment, many after as little as 14 days. In two thirds of cases PgR became undetectable after three months of therapy. In contrast, the most consistent effect on PgR seen with neoadjuvant tamoxifen treatment, was an increase in the PgR expression [See table 25]. [150]

Treatment	Decrease in PgR	No change in PgR	Increase in PgR
Letrozole	44 (73%)	16 (27%)	0
Tamoxifen (ref)	12 (23%)	13 (25%)	27 (52%)

Table 25: Comparison of the effects of Letrozole and Tamoxifen on PgR expression (tamoxifen data from previous Edinburgh series [150]. Percentages refer to % of patients showing change in PgR.

Makris et al reported that 41% of patients in their series showed an increase in PgR expression after 14 days neoadjuvant treatment with tamoxifen [125]. Another 17% showed no change in PgR expression with treatment. Therefore, tamoxifen and letrozole have significantly different effects on PgR expression.

After three months treatment with letrozole, 82% of cases in this series showed a marked decrease in PgR expression. In two thirds of cases, this was a decrease to zero. Decreases in staining were seen even in the absence of pathological response. This is in contrast to changes seen after treatment with neoadjuvant tamoxifen where the most common change was an increase in PgR expression.

Despite significant decreases in expression of PgR being observed as early as 14 days into treatment with letrozole, it was not possible to detect any consistent pattern in PgR changes between clinical or pathological responding and non-responding tumours. The decrease in PgR was seen in tumours which responded to treatment and tumours which did not. Therefore, lack of clinical or pathological response is not because the tumour fails to recognize letrozole as an oestrogen depriving therapy.

The initial PgR status was not predictive of the clinical or pathological response to treatment. Some tumours that were PgR negative exhibited clear evidence of response. The effect of letrozole on PgR is clear evidence of a different mode of action from tamoxifen which has variable effects on PgR expression including increased expression.

Looking at PgR expression in the PO24 study, the authors also noted that letrozole induced profound down regulation of PgR. In contrast, tamoxifen demonstrated a mixed agonist/ antagonist effect on PgR. This confirmed the profound differences in the molecular effects of the two drugs. The degree of PgR suppression was greater for tumours in which a mammographic response was documented. The authors speculated that PgR expression in post-treatment samples could be a useful biomarker for the effectiveness of oestrogen deprivation therapy [129]. However, this was not confirmed in this study.

In the PO24 study, peak response rates were seen to occur in tumours that scored PgR Allred 4 or 5 with both tamoxifen and letrozole. Both high and low levels of PgR expression were associated with a lower chance of response compared with intermediate scores [141]. Unlike ER, the relationship between PgR Allred score and response did not fit a linear model. The model that best fitted the data was an inverse V-shaped model with the peak response to letrozole seen at PgR score 5 and to tamoxifen seen at PgR 4.

This relationship was more complex than expected from prior information about the predictive properties of PgR in breast cancer [151]. The authors acknowledged that it had been assumed that the relationship between PgR expression and response would be linear as with ER since PgR is generally accepted as a marker for oestrogen dependent cancers with a functional ER because PgR requires activated ER for expression. This conventional model explains the initial increase in response rates seen from PgR Allred

score 0 to 5. However, it does not explain the subsequent decline in response seen from PgR score 6 to 8. The authors speculated that PgR rich tumours may be associated with sufficiently high levels of aromatase activity or hypersensitivity to oestrogen that they might blunt the efficacy of oestrogen deprivation therapy. Another theory is that it is possible that PgR rich tumours carry a mutation in the ER which cause cancer cells to become hypersensitive to oestrogen [141]. It is also important to remember that just because a tumour is oestrogen sensitive it doesn't mean that it will definitely respond to treatment. In almost all cases, the genes that change most will not consistently predict response because at most only 70-80% of tumours will respond to treatment. The ATAC study reported a better response for anastrozole compared with tamoxifen, particularly in patients who were ER positive and PgR negative[94]. However, in contrast, BIG 1-98 showed the best outcome to be in those patients who were both ER and PgR positive[93]. Therefore there is confusion about the effect of AIs on the different tumour phenotypes in the available literature in the adjuvant setting.

The Oxford overview analysis did not show PgR to be a useful predictor for the adjuvant benefit of tamoxifen. [75]. It may be that tamoxifen and letrozole have different relationships to PgR expression because they have a different mode of action. This means that the value of PgR as a predictive biomarker is uncertain and more work needs to be undertaken in order to fully understand the mechanism and implications of the effect seen on PgR. The difference in tumour phenotype after treatment may have clinical relevance in terms of resistance to treatment and in making decisions about

further treatment choices. Aromatase inhibitors have been shown to have therapeutic benefit in patients whose tumours have acquired resistance to tamoxifen [152].

4.7 Effects on proliferation

Pre-treatment scores for Ki67 were similar in responders and non-responders so baseline level of proliferation did not appear to predict response. It has been suggested that tumours which have higher proliferation are more likely to respond to neoadjuvant chemotherapy [146] but this does not seem to be the case for neoadjuvant endocrine therapy.

Treatment with letrozole for as short a period as 14 days was associated with highly significant decreases in proliferation in all tumour sub groups in this study. However, there was no significant difference between clinical responders and non-responders. Interestingly, there was a significant difference between pathological responders and non-responders implying that morphological features of response might be more accurate than clinical ones at identifying responders to neoadjuvant endocrine therapy.

After three months of treatment with letrozole, there was no significant difference between clinical responders and non-responders. The mean reduction in proliferation for clinical responders was 75% and 66% for non-responders.

The consistent decreases in proliferation with letrozole and also described for the other aromatase inhibitors, was not the pattern consistently seen with tamoxifen [145;153]. Tamoxifen treatment was also associated with an increase in proliferation markers,

particularly in non responding tumours [148]. These changes may reflect differences in the mechanism of action between tamoxifen and the aromatase inhibitors.

In this study, 12 out of 13 clinical non-responders showed a reduction in proliferation and only one tumour showed an increase in proliferation of 43% after treatment. Interestingly, although the majority of clinical responders showed a drop in proliferation with treatment, there were also three patients in that group whose tumours increased in proliferation over the treatment period (mean increase 19%). Therefore, change in proliferation does not appear able to discriminate between tumours likely to respond and those not likely to.

In the PO24 study [41], the fall in Ki67 was significantly greater in responders than non-responders ($p=0.025$ by Mann-Whitney test) confirming the effect of response on tumour proliferation. However, the response modality used in PO24 to categorise responders and non-responders was the change in caliper tumour size which is different to the US response used in this study. Table 26 shows the effect of letrozole and tamoxifen on Ki67 in PO24.

		No	% decrease in the geometrical mean Ki67	Wilcoxon signed rank test P
All cases	Letrozole	93	87	<0.0001
	Tamoxifen	92	75	<0.0001

Table 26: Effect of letrozole and tamoxifen on Ki67 [41]

For all cases, letrozole had a significantly greater effect on proliferation than tamoxifen (p=0.0009). In another study looking at proliferation in patients treated with neoadjuvant tamoxifen, Makris et al showed that 79% of tamoxifen treated tumours showed a decrease in Ki67 after 14 days treatment [125]. Interestingly, 100% of responders showed a decrease in proliferation, while a third of non-responders showed an increase in proliferation after treatment with tamoxifen.

The effects of letrozole on Ki67 expression were striking implying that one important effect of letrozole is to remove tumour cells from the cycle of division. However, whether or not this translates to a clinical or pathological response is more complex and involves other factors. Proliferation is likely to be oestrogen dependent. Therefore, any agent which reduces oestrogen should consequently reduce proliferation. However, clinical response is clearly more complex than simply switching off proliferation. In terms of correlating changes in proliferation with clinical response, any factor which decreases in such a large percentage of patients is unlikely to be helpful in predicting

response. The practical implication is that, on an individual basis, measurement of Ki67 is not helpful in predicting or monitoring response.

This study did not look at the effects of letrozole on cell death for several reasons. Firstly, the estimation of cell apoptosis is laborious and notoriously difficult to estimate accurately. Secondly, studies which have attempted to compare apoptosis rates between AIs and tamoxifen, for example the IMPACT study, have not found any significant difference[113].

4.8 HER 2 (erbB2)

It has been suggested that the over expression of HER1 or HER2 may be associated with an increase in the likelihood of response to letrozole compared to tamoxifen. In the PO24 study, letrozole's ability to suppress the proliferation of ER+ breast cancer cells was not affected by HER 1 or HER 2 over-expression [41]. In contrast, HER 1 or HER 2 positive tumours exhibited evidence of resistance to the anti-proliferative effects of tamoxifen (see table 27).

		Number of patients	% decrease in the geometrical mean Ki67	Wilcoxon signed rank test p
Her1/2}	Letrozole	78	86	<0.0001
negative}	Tamoxifen	75	79	<0.0001
Her 1/2 }	Letrozole	15	88	0.0166
positive}	Tamoxifen	17	45	NS

Table 27: Effect of letrozole and tamoxifen on Ki67 according to HER 1/2 status [129]

There was an 88% response rate in the HER1 or 2 positive group treated with letrozole compared with 21% when treated with tamoxifen. It may be that these patients are resistant to tamoxifen but respond to aromatase inhibitors. This may explain why the

aromatase inhibitors appear to have improved efficacy in direct comparison with tamoxifen.

In this current series, positive membrane staining for HER2 over-expression was detected in 12/63 (19%) of tumours which is slightly higher than the approximately 10% that might be expected in a group of ER rich tumours. Neoadjuvant letrozole in this series of ER + breast cancers was equally effective in both HER2 positive and negative tumours. It reduced tumour volume at three months by at least 60% in both groups. Looking at the whole series of letrozole treated patients, at 3 months by WHO criteria 106/154 (69%) Her 2 negative and 11/18 (61%) Her 2 positive tumours had a clinical response ($p=0.506$, Fisher's exact test). In addition there was no significant difference in the reduction in proliferation between groups. The efficacy of letrozole does not therefore appear to be influenced by Her 2 status.

4.9 Optimum duration of neoadjuvant endocrine therapy

Randomised studies of neoadjuvant aromatase inhibitors to date have treated patients for either three or four months. By that time many patients' tumours have responded sufficiently to downstage surgery from mastectomy to breast conserving surgery, but some remain inoperable or still require mastectomy. Few studies have to date examined this issue in detail.

In a series of 100 elderly patients treated with tamoxifen, Dixon et al suggested that three months was long enough to distinguish between responders and non responders and to achieve optimal tumour shrinkage preoperatively [118]. Maximal response may, however, take considerably longer than three months so the optimal period of treatment depends on initial tumour size and the aim of the neoadjuvant therapy. If the aim is to downstage the tumour to allow breast conserving surgery to be performed, this can be achieved in the majority of patients in three to four months. There has been concern that, when treating patients for longer than three months, tumours which initially responded may acquire resistance and begin to progress.

This study looked in more detail at extending the use of neoadjuvant letrozole for longer periods of time. 30% of patients in the study continued on letrozole for more than three months. This was done for a variety of reasons. For example, they were deemed unfit for surgery, refused surgery, had responded but still required mastectomy or were still

inoperable after three months of treatment. One patient who was responding at three months had disease progression at 12 months but the rest continued to respond to letrozole over the treatment period. For up to a year, letrozole continued to be effective at reducing the size of the tumour and few tumours stopped responding to treatment in this timescale. Therefore, patients whose tumours are responding to letrozole at three months can expect further reduction in tumour volume with continued treatment. Fewer patients appeared to acquire resistance to letrozole over a 12 month period than did with tamoxifen.

These findings suggest that neoadjuvant letrozole can be used safely for up to 12 months to achieve optimal tumour shrinkage. It has to be borne in mind that similar responses were seen with tamoxifen with a mean period of response of 24 months and that only a small percentage of patients were long term responders not requiring surgery by 10 years. It is likely to remain difficult to identify which patients are going to be the long term responders.

There is therefore no identified optimum duration for use of neoadjuvant letrozole although it appears it can be used safely in women with responsive cancers for up to 12 months.

4.10 Completeness of tumour excision and incidence of local recurrence

Reducing the size of large or inoperable tumours in patients with early stage breast cancer has been shown to increase the success of breast-conserving surgery (BCS) [101;154]. However, several series have described relatively high rates of positive margins after neoadjuvant treatment with chemotherapy. In the Royal Marsden series, 28% of patients who had breast conserving surgery had positive margins [30]. In another series of patients with locally advanced breast cancer treated with neoadjuvant chemotherapy, 62.5% of patients had multiple foci of tumour remaining after wide local excision [155]. This may partly be due to the nature of response of the tumour to the chemotherapy. In the Milan series, 16% of the 227 cases had evidence of tumour multifocality within the wide local excision specimen [156].

In this study, when assessing the residual wide local excision samples histologically, the nature of response to neoadjuvant letrozole appeared different from the response previously described after chemotherapy. The whole tumour appeared to have shrunk concentrically which is similar to the response to aromatase inhibitors previously described [118]. However, there was a 13% positive margin rate that required further surgery to be performed. Several of these cases were invasive lobular carcinomas which were found to be extremely difficult to assess in terms of clinical response to treatment. Since approximately half of invasive lobular carcinomas have involved margins after

wide local excision (WLE) it was felt that these tumours were unsuitable for this type of neoadjuvant endocrine therapy. Since they almost always require mastectomy for adequate surgical treatment they were latterly excluded from the study.

After preoperative chemotherapy, local recurrence rates following WLE have been reported to vary between 3.5% and 6% (after median three year follow up) [156;157]. However, in one study there was a 22% recurrence rate in the patients who had a mastectomy after preoperative chemotherapy because their tumour had not responded enough to allow BCS [156].

There is little available data on recurrence after BCS following neoadjuvant endocrine therapy. Some data from patients previously treated in Edinburgh with tamoxifen and Aromatase inhibitors are presented in table 28.

Agent	No. of patients	No. with No XRT	No. with Local recurr	No. with XRT	No. with local recurr	Median F/up (mths)
Tamoxifen	47	13	4	34	0	84
Letrozole	34	10	4	24	1	70
Anastrozole	21	0	0	21	1	51
Exemestane	10	4	1	6	0	42
Total	112	27	9	85	2	62

Table 28: Local recurrence in patients treated with neoadjuvant endocrine therapy in relation to whether or not they had post operative radiotherapy (XRT)

As can be seen from table 28, the rate of local recurrence is much higher in those patients who did not receive radiotherapy after their breast conserving surgery. There was a 33% local recurrence rate in the patients who did not receive XRT vs a 2.4% local recurrence rate in those that did [118]. For comparison, local recurrence rates for patients treated in the standard fashion by surgery followed by systemic therapy have been reported to be 2.7% in one series [157].

With a mean follow up period of 39 months (4-58), the majority of local recurrences in this study should have occurred. However, there have been only seven local recurrences to date (5% of patients), several in patients with very aggressive advanced disease. Three of the patients who developed local recurrence have been treated and remain alive and well. Of the four who died, three had evidence of distant metastasis in addition to their local recurrence. The mean time to recurrence for these patients was 22 months (range 9-39 months).

4.11 Survival

With a mean follow up period of 39 months (4-58), it is still a relatively short period of time to look at disease free (DFS) and overall survival. In this series, 42 patients died (33% of the group). 23 of these deaths were from breast cancer and 18 from other causes giving a disease free survival of 82% and an overall survival rate of 67%.

It is difficult to compare the DFS and the overall survival of this group with a series of patients treated with adjuvant endocrine therapy. Despite having locally advanced breast cancer, many of the patients treated with neoadjuvant endocrine therapy will die from other causes as a result of their comorbidity. DFS and OS have been reported in other studies to be similar in patients treated with neoadjuvant chemotherapy preoperatively and in patients treated with surgery followed by systemic therapy [27-29;32].

Cameron et al reported a randomised trial comparing oestrogen receptor directed primary systemic therapy with conventional therapy in operable breast cancer and showed no difference in survival between groups treated with neoadjuvant endocrine or chemotherapy and those having follow up systemic therapy after surgery [158].

It is of interest that a significant correlation was seen between the percentage drop in Ki67 after three months of treatment with letrozole and disease free survival ($p=0.007$). Patients who have a complete pathological response to neoadjuvant chemotherapy have

been shown to have improved disease free survival compared to patients who do not. However, complete pathological response to neoadjuvant endocrine therapy is rare so it is difficult to relate response to treatment to prognosis. It may be that Ki67 has potential to be used as a marker of response to endocrine therapy which correlates with long term survival. However, at present it is not helpful and can not be used to predict outcome on an individual patient basis.

Section 5: Conclusions

This thesis has examined the response of breast cancer to neoadjuvant endocrine therapy using several clinical methods and biological markers. In particular it has focused on changes in tumour phenotype after two weeks and three months of neoadjuvant treatment with the aromatase inhibitor letrozole.

Neoadjuvant endocrine therapy with letrozole was extremely successful in the selected group of patients with ER rich tumours that took part in this study. Two thirds of patients showed an overall response to treatment and two thirds of patients had their surgery down-staged from mastectomy to breast conserving surgery.

The speed of response to treatment was variable. In general, it appeared somewhat slower than with neoadjuvant chemotherapy but interestingly response to treatment with neoadjuvant letrozole continued for 12 months or longer. Tumour response was seen quickly using ultrasound scanning with responses being evident after as little time as four weeks. By six weeks, it was usually possible to accurately predict whether or not breast conserving surgery would be possible after three months of treatment.

A major issue in the use of neoadjuvant endocrine therapy is the selection of patients who are likely to benefit most from treatment. This study has shown that patients with ER rich tumours (Allred score 7 or 8) are the ones that are likely to respond best. Tumours that score ER 8 were seen to achieve a greater percentage reduction in tumour volume than those that were ER 7. It has also demonstrated that patients will still

respond well to treatment even if they over express HER2 as long as their tumour is ER rich.

Another aim of this study was to try to predict the degree of clinical response to neoadjuvant treatment to allow the targeted selection of patients. Apart from ER, no other baseline biological marker was shown to accurately predict the tumours most likely to respond to treatment. There was a small decrease in ER expression over the treatment period. This was probably related to fixation and therefore not clinically relevant. Significant decreases in expression of PgR were observed at 14 days in all patient groups but this did not correlate with clinical or pathological tumour response.

In the biological markers subgroup of 62 patients, 78% patients had a clinical response (>50% reduction in tumour volume at three months by serial ultrasound) and 75% of tumours displayed evidence of a pathological response (decreased cellularity / increased fibrosis).

Proliferation has been shown to decrease during treatment with the aromatase inhibitors letrozole and anastrozole, and occurs as early in treatment as 10-14 days. In this study, pre-treatment scores for Ki67 were similar in responders and non-responders whether assessed clinically or pathologically. Treatment was associated with highly significant Ki67 decreases in all tumour sub-groups (all at least $p< 0.005$ by paired Wilcoxon rank test) at 14 days. However, levels of Ki67 14 days into treatment were not significantly different in clinical responders and non responders.

When correlating the decrease in proliferation with pathological response, Ki67 expression at 14 days was significantly higher in tumours which subsequently failed to show morphological evidence of response. These changes in morphology are associated with differences in tumour behaviour and the magnitude of decrease in Ki67 after three months has been shown to correlate with long-term outcome.

Proliferation as assessed by Ki67 has been shown to reduce significantly after a short period of treatment but this does not directly correlate with clinical response over the three month treatment period. However, correlation with morphological response to treatment was demonstrated. The fresh tissue collected as part of this study is currently being analysed by microarray and will hopefully suggest possible avenues for other markers which may prove more helpful in predicting response to neoadjuvant endocrine treatment on an individual patient basis.

This study has shown letrozole decreases the expression of both PgR and Ki67 in a striking manner. This reflects the powerful anti-oestrogenic and anti-proliferative potential of the drug. The intermediary mechanisms by which letrozole achieves a clinical or pathological response remain unclear.

Section 6: Future work

There are many aspects of this study which are ongoing and which will provide much more information after a longer period of follow up. There is little information on the local recurrence and long term survival of patients treated with neoadjuvant letrozole. While initial results for the use of neoadjuvant tamoxifen were promising, the long term local disease control proved poor. It will be interesting to see if this is the case with letrozole in the small group of patients who continue on the drug, having had no other treatment for their breast cancer.

At the time of taking serial tumour samples, in addition to paraffin embedded material, extra core biopsies were taken and fresh frozen in liquid nitrogen to allow microarray work to be performed. The preliminary results are available and some can be seen in the papers and abstracts at the end of this thesis. While Ki67 has been shown to be a useful marker of tumour proliferation, it does not help to predict on an individual basis which tumours are likely to respond to neoadjuvant letrozole after 14 days. It would be useful to identify markers which might be able to do this and therefore help to guide patient selection for this treatment.

There is also no clear information on the effect of aromatase inhibitors in tumours which express lower levels of ER (Allred score 2-6). Some data from PO24 suggested that letrozole but not tamoxifen may have a role in treating these patients. However, the numbers of patients included were small and more work needs to be done to clarify this.

Section 7: References

Reference List

1. National Statistics online accessed Feb 2004. www.statistics.gov.uk . 2006.
Ref Type: Electronic Citation

2. isdscotland. www.isdscotland.com . 2004.
Ref Type: Electronic Citation

3. Forbes JF. The incidence of breast cancer: the global burden, public health considerations. *Semin.Oncol.* 1997;**24**:S1.

4. Shibuya K, Mathers CD, Boschi-Pinto C, Lopez AD, Murray CJ. Global and regional estimates of cancer mortality and incidence by site: II. Results for the global burden of disease 2000. *BMC.Cancer* 2002;**2**:37.

5. Donegan WL,.Redlich PN. Breast cancer in men. *Surg Clin.North Am.* 1996;**76**:343-63.

6. Henderson IC. What can a woman do about her risk of dying of breast cancer? *Curr.Probl.Cancer* 1990;**14**:161-230.

7. Yost K, Perkins C, Cohen R, Morris C, Wright W. Socioeconomic status and breast cancer incidence in California for different race/ethnic groups. *Cancer Causes Control* 2001;**12**:703-11.

8. Altaha R, Reed E, Abraham J. Breast and ovarian cancer genetics and prevention. *W.V.Med.J* 2003;**99**:187-91.

9. McPherson K, Steel CM, Dixon JM. ABC of breast diseases. Breast cancer-epidemiology, risk factors, and genetics. *BMJ* 2000;**321**:624-8.

10. Thomas DB,.Noonan EA. Breast cancer and prolonged lactation. The WHO Collaborative Study of Neoplasia and Steroid Contraceptives. *Int.J Epidemiol.* 1993;**22**:619-26.

11. Westhoff CL. Breast cancer risk: perception versus reality. *Contraception* 1999;**59**:25S-8S.

12. Dixon JM. Hormone replacement therapy and the breast. *Surg Oncol.* 2003;**12**:251-63.

13. Beral V. Breast cancer and hormone-replacement therapy in the Million Women Study. *Lancet* 2003;**362**:419-27.

14. Hunter DJ,.Willett WC. Nutrition and breast cancer. *Cancer Causes Control* 1996;7:56-68.

15. Holmberg L, Baron JA, Byers T, Wolk A, Ohlander EM, Zack M *et al.* Alcohol intake and breast cancer risk: effect of exposure from 15 years of age. *Cancer Epidemiol.Biomarkers Prev.* 1995;**4**:843-7.

16. Ali S,.Coombes RC. Endocrine-responsive breast cancer and strategies for combating resistance. *Nat.Rev.Cancer* 2002;**2**:101-12.

17. Purushotham AD, Pain SJ, Miles D, Harnett A. Variations in treatment and survival in breast cancer. *Lancet Oncol.* 2001;**2**:719-25.

18. Yiangou C, Davis J, Livni N, Barrett NK, Sinnett HD. Diagnostic role of cytology in screen-detected breast cancer. *Br J Surg* 1996;**83**:816-9.

19. Jacobs TW, Siziopikou KP, Prioleau JE, Raza S, Baum JK, Hayes DF *et al.* Do prognostic marker studies on core needle biopsy specimens of breast carcinoma accurately reflect the marker status of the tumor? *Mod.Pathol.* 1998;**11**:259-64.

20. Morris AD, Morris RD, Wilson JF, White J, Steinberg S, Okunieff P *et al.* Breast-conserving therapy vs mastectomy in early-stage breast cancer: a meta-analysis of 10-year survival. *Cancer J Sci.Am.* 1997;**3**:6-12.

21. Effects of radiotherapy and surgery in early breast cancer. An overview of the randomized trials. Early Breast Cancer Trialists' Collaborative Group. *N.Engl.J Med.* 1995;**333**:1444-55.

22. Bundred NJ, Morgan DA, Dixon JM. ABC of breast diseases. Management of regional nodes in breast cancer. *BMJ* 1994;**309**:1222-5.

23. Thompson AM, Pinder SE. Prognostic factors. In Dixon JM, ed. *ABC of Breast Diseases*, pp 77-80. London: Blackwell Publishing, 2006.

24. Blamey RW. The design and clinical use of the Nottinham Prognostic Index in breast cancer. *The Breast* 1996;**5**:156-7.

25. Blamey R and et al. Nottingham Prognostic Index. The Breast . 1996.
Ref Type: Abstract

26. Systemic treatment of early breast cancer by hormonal, cytotoxic, or immune therapy. 133 randomised trials involving 31,000 recurrences and 24,000 deaths among 75,000 women. Early Breast Cancer Trialists' Collaborative Group. *Lancet* 1992;**339**:1-15.

27. Kurtz JM. Should surgery remain the initial treatment of "operable" breast cancer? *Eur.J Cancer* 1991;**27**:1539-42.

28. Fisher B, Bryant J, Wolmark N, Mamounas E, Brown A, Fisher ER *et al.* Effect of preoperative chemotherapy on the outcome of women with operable breast cancer. *J Clin.Oncol.* 1998;**16**:2672-85.

29. Mauriac L, Durand M, Avril A, Dilhuydy JM. Effects of primary chemotherapy in conservative treatment of breast cancer patients with operable tumors larger than 3 cm. Results of a randomized trial in a single centre. *Ann.Oncol.* 1991;**2**:347-54.

30. Mauriac L, MacGrogan G, Avril A, Durand M, Floquet A, Debled M *et al.* Neoadjuvant chemotherapy for operable breast carcinoma larger than 3 cm: a unicentre randomized trial with a 124-month median follow-up. Institut Bergonie Bordeaux Groupe Sein (IBBGS). *Ann.Oncol.* 1999;**10**:47-52.

31. Robertson JF, Ellis IO, Elston CW, Blamey RW. Mastectomy or tamoxifen as initial therapy for operable breast cancer in elderly patients: 5-year follow-up. *Eur.J Cancer* 1992;**28A**:908-10.

32. Semiglazov VF, Topuzov EE, Bavli JL, Moiseyenko VM, Ivanova OA, Seleznev IK *et al.* Primary (neoadjuvant) chemotherapy and radiotherapy compared with primary radiotherapy alone in stage IIb-IIIa breast cancer. *Ann.Oncol.* 1994;**5**:591-5.

33. Gazet JC, Ford HT, Coombes RC, Bland JM, Sutcliffe R, Quilliam J *et al.* Prospective randomized trial of tamoxifen vs surgery in elderly patients with breast cancer. *Eur.J Surg Oncol.* 1994;**20**:207-14.

34. Mustacchi G, Milani S, Pluchinotta A, De Matteis A, Rubagotti A, Perrota A. Tamoxifen or surgery plus tamoxifen as primary treatment for elderly patients with operable breast cancer: The G.R.E.T.A. Trial. Group for Research on Endocrine Therapy in the Elderly. *Anticancer Res* 1994;**14**:2197-200.

35. Bates T, Riley DL, Houghton J, Fallowfield L, Baum M. Breast cancer in elderly women: a Cancer Research Campaign trial comparing treatment with tamoxifen and optimal surgery with tamoxifen alone. The Elderly Breast Cancer Working Party. *Br J Surg* 1991;**78**:591-4.

36. Thomas E, Holmes FA, Smith TL, Buzdar AU, Frye DK, Fraschini G *et al.* The use of alternate, non-cross-resistant adjuvant chemotherapy on the basis of pathologic response to a neoadjuvant doxorubicin-based regimen in women with operable breast cancer: long-term results from a prospective randomized trial. *J Clin Oncol* 2004;**22**:2294-302.

37. Polychemotherapy for early breast cancer: an overview of the randomised trials. Early Breast Cancer Trialists' Collaborative Group. *Lancet* 1998;**352**:930-42.

38. Keen JC, Dixon JM, Miller EP, Cameron DA, Chetty U, Hanby A *et al.* The expression of Ki-S1 and BCL-2 and the response to primary tamoxifen therapy in elderly patients with breast cancer. *Breast Cancer Res Treat.* 1997;**44**:123-33.

39. van Dalsen AD,.de Vries JE. Treatment of breast cancer in elderly patients. *J Surg Oncol.* 1995;**60**:80-2.

40. Fennessy M, Bates T, MacRae K, Riley D, Houghton J, Baum M. Late follow-up of a randomized trial of surgery plus tamoxifen versus tamoxifen alone in women aged over 70 years with operable breast cancer. *Br J Surg* 2004;**91**:699-704.

41. Eiermann W, Paepke S, Appfelstaedt J, Llombart-Cussac A, Eremin J, Vinholes J *et al.* Preoperative treatment of postmenopausal breast cancer patients with letrozole: A randomized double-blind multicenter study. *Ann.Oncol.* 2001;**12**:1527-32.

42. Sader C, Ingram D, Hastrich D. Management of breast cancer in the elderly by complete local excision and tamoxifen alone. *Aust.N.Z.J Surg* 1999;**69**:790-3.

43. Wanebo HJ, Cole B, Chung M, Vezeridis M, Schepps B, Fulton J *et al.* Is surgical management compromised in elderly patients with breast cancer? *Ann.Surg* 1997;**225**:579-86.

44. Silliman RA, Balducci L, Goodwin JS, Holmes FF, Leventhal EA. Breast cancer care in old age: what we know, don't know, and do. *J Natl.Cancer Inst.* 1993;**85**:190-9.

45. Extermann M, Balducci L, Lyman GH. What threshold for adjuvant therapy in older breast cancer patients? *J Clin Oncol* 2000;**18**:1709-17.

46. Yancik R, Wesley MN, Ries LA, Havlik RJ, Edwards BK, Yates JW. Effect of age and comorbidity in postmenopausal breast cancer patients aged 55 years and older. *JAMA* 2001;**285**:885-92.

47. Petrisek AC, Laliberte LL, Allen SM, Mor V. The treatment decision-making process: age differences in a sample of women recently diagnosed with nonrecurrent, early-stage breast cancer. *Gerontologist* 1997;**37**:598-608.

48. Diab SG, Elledge RM, Clark GM. Tumor characteristics and clinical outcome of elderly women with breast cancer. *J Natl.Cancer Inst.* 2000;**92**:550-6.

49. Ganz PA, Lee JJ, Sim MS, Polinsky ML, Schag CA. Exploring the influence of multiple variables on the relationship of age to quality of life in women with breast cancer. *J Clin Epidemiol.* 1992;**45**:473-85.

50. Sandison AJ, Gold DM, Wright P, Jones PA. Breast conservation or mastectomy: treatment choice of women aged 70 years and older. *Br J Surg* 1996;**83**:994-6.

51. Wyld L,.Reed MW. The need for targeted research into breast cancer in the elderly. *Br J Surg* 2003;**90**:388-99.

52. Diab SG, Elledge RM, Clark GM. Tumor characteristics and clinical outcome of elderly women with breast cancer. *J Natl.Cancer Inst.* 2000;**92**:550-6.

53. Berg JW,.Hutter RV. Breast cancer. *Cancer* 1995;**75**:257-69.

54. Allan SG, Rodger A, Smyth JF, Leonard RC, Chetty U, Forrest AP. Tamoxifen as primary treatment of breast cancer in elderly or frail patients: a practical management. *Br Med.J (Clin.Res Ed)* 1985;**290**:358.

55. Gaskell DJ, Hawkins RA, Sangsterl K, Chetty U, Forrest AP. Relation between immunocytochemical estimation of oestrogen receptor in elderly patients with primary breast cancer and response to tamoxifen. *Lancet* 1989;**1**:1044-6.

56. Gaskell DJ, Hawkins RA, de Carteret S, Chetty U, Sangster K, Forrest AP. Indications for primary tamoxifen therapy in elderly women with breast cancer. *Br J Surg* 1992;**79**:1317-20.

57. Bradbeer JW,.Kyngdon J. Primary treatment of breast cancer in elderly women with Tamoxifen. *Clin Oncol* 1983;**9**:31-4.

58. Preece PE, Wood RA, Mackie CR, Cuschieri A. Tamoxifen as initial sole treatment of localised breast cancer in elderly women: a pilot study. *Br Med J (Clin Res Ed)* 1982;**284**:869-70.

59. Horobin JM, Preece PE, Dewar JA, Wood RA, Cuschieri A. Long-term follow-up of elderly patients with locoregional breast cancer treated with tamoxifen only. *Br J Surg* 1991;**78**:213-7.

60. Low SC, Dixon AR, Bell J, Ellis IO, Elston CW, Robertson JF *et al.* Tumour oestrogen receptor content allows selection of elderly patients with breast cancer for conservative tamoxifen treatment. *Br J Surg* 1992;**79**:1314-6.

61. Coombes RC, Powles TJ, Berger U, Wilson P, McClelland RA, Gazet JC *et al.* Prediction of endocrine response in breast cancer by immunocytochemical detection of oestrogen receptor in fine-needle aspirates. *Lancet* 1987;**2**:701-3.

62. Bates, T., Fennessy M, Riley DL, Baum M, and Houghton J. Breastcancer in the elderly: surgery improves survival. Eur.J Cancer 37 (suppl 5). 2001.
Ref Type: Abstract

63. Kenny FS, Robertson JFR, Ellis IO, Elston CW, Blamey RW. Long-term follow up of elderly patients randomized to primary tamoxifen or wedge mastectomy as initial therapy for operable breast cancer. *Breast* 1998;7:335-9.

64. Kesseler HJ,.Seton JZ. The treatment of operable breast cancer in the elderly female. *Am J Surg* 1978;**135**:664-6.

65. Hunt KE, Fry DE, Bland KI. Breast carcinoma in the elderly patient: an assessment of operative risk, morbidity and mortality. *Am J Surg* 1980;**140**:339-42.

66. Grube BJ, Hansen NM, Ye W, Herlong T, Giuliano AE. Surgical management of breast cancer in the elderly patient. *Am J Surg* 2001;**182**:359-64.

67. Gajdos C, Tartter PI, Bleiweiss IJ, Lopchinsky RA, Bernstein JL. The consequence of undertreating breast cancer in the elderly. *J Am Coll.Surg* 2001;**192**:698-707.

68. Jubelirer SJ,.Larzo CR. The treatment of breast cancer in the elderly: a community hospital experience. *W.V.Med J* 1998;**94**:329-31.

69. Cerrotta A, Lozza L, Kenda R, Gardani G, Galante E, Zucali R. Current controversies in the therapeutic approach to early breast cancer in the elderly. *Rays* 1997;**22**:66-8.

70. Yeh S, Tan LR, O'Connell TX. Segmental mastectomy and tamoxifen alone provide adequate locoregional control of breast cancer in elderly women. *Am Surg* 1997;**63**:854-7.

71. Nemoto T, Patel JK, Rosner D, Dao TL, Schuh M, Penetrante R. Factors affecting recurrence in lumpectomy without irradiation for breast cancer. *Cancer* 1991;**67**:2079-82.

72. Kuehn T, Klauss W, Darsow M, Regele S, Flock F, Maiterth C *et al.* Long-term morbidity following axillary dissection in breast cancer patients--clinical assessment, significance for life quality and the impact of demographic, oncologic and therapeutic factors. *Breast Cancer Res Treat.* 2000;**64**:275-86.

73. Kissin MW, Querci della RG, Easton D, Westbury G. Risk of lymphoedema following the treatment of breast cancer. *Br J Surg* 1986;**73**:580-4.

74. Randomized trial of two versus five years of adjuvant tamoxifen for postmenopausal early stage breast cancer. Swedish Breast Cancer Cooperative Group. *J Natl.Cancer Inst.* 1996;**88**:1543-9.

75. Tamoxifen for early breast cancer: an overview of the randomised trials. Early Breast Cancer Trialists' Collaborative Group. *Lancet* 1998;**351**:1451-67.

76. Controlled trial of tamoxifen as adjuvant agent in management of early breast cancer. Interim analysis at four years by Nolvadex Adjuvant Trial Organisation. *Lancet* 1983;**1**:257-61.

77. Mouridsen H, Palshof T, Patterson J, Battersby L. Tamoxifen in advanced breast cancer. *Cancer Treat.Rev.* 1978;**5**:131-41.

78. Kiang DT,.Kennedy BJ. Tamoxifen (antiestrogen) therapy in advanced breast cancer. *Ann.Intern.Med.* 1977;**87**:687-90.

79. Brodie AM,.Njar VC. Aromatase inhibitors and breast cancer. *Semin.Oncol.* 1996;**23**:10-20.

80. Scott LJ,.Wiseman LR. Exemestane. *Drugs* 1999;**58**:675-80.

81. Geisler J, Haynes B, Anker G, Dowsett M, Lonning PE. Influence of letrozole and anastrozole on total body aromatization and plasma estrogen levels in postmenopausal breast cancer patients evaluated in a randomized, cross-over study. *J Clin Oncol* 2002;**20**:751-7.

82. Geisler J, King N, Anker G, Ornati G, Di Salle E, Lonning PE *et al.* In vivo inhibition of aromatization by exemestane, a novel irreversible aromatase inhibitor, in postmenopausal breast cancer patients. *Clin.Cancer Res* 1998;**4**:2089-93.

83. Dowsett M, Geisler J, and Haynes BP. Letrozole achieves more complete inhibition of whole body aromatisation than anastrozole. Eur.J Cancer 36(S88). 2001.
Ref Type: Abstract

84. Geisler J, King N, Dowsett M, Ottestad L, Lundgren S, Walton P *et al.* Influence of anastrozole (Arimidex), a selective, non-steroidal aromatase inhibitor, on in vivo aromatisation and plasma oestrogen levels in postmenopausal women with breast cancer. *Br J Cancer* 1996;**74**:1286-91.

85. Yates RA, Dowsett M, Fisher GV, Selen A, Wyld PJ. Arimidex (ZD1033): a selective, potent inhibitor of aromatase in postmenopausal female volunteers. *Br J Cancer* 1996;**73**:543-8.

86. Lonning PE, Bajetta E, Murray R, Tubiana-Hulin M, Eisenberg PD, Mickiewicz E *et al.* Activity of exemestane in metastatic breast cancer after failure of nonsteroidal aromatase inhibitors: a phase II trial. *J Clin.Oncol.* 2000;**18**:2234-44.

87. Buzdar AU, Jonat W, Howell A, Jones SE, Blomqvist CP, Vogel CL *et al.* Anastrozole versus megestrol acetate in the treatment of postmenopausal women with advanced breast carcinoma: results of a survival update based on a combined analysis of data from two mature phase III trials. Arimidex Study Group. *Cancer* 1998;**83**:1142-52.

88. Dombernowsky P, Smith I, Falkson G, Leonard R, Panasci L, Bellmunt J *et al.* Letrozole, a new oral aromatase inhibitor for advanced breast cancer: double-blind randomized trial showing a dose effect and improved efficacy and tolerability compared with megestrol acetate. *J Clin.Oncol.* 1998;**16**:453-61.

89. Kaufmann M, Bajetta E, Dirix LY, Fein LE, Jones SE, Zilembo N *et al.* Exemestane is superior to megestrol acetate after tamoxifen failure in postmenopausal women with advanced breast cancer: results of a phase III randomized double-blind trial. The Exemestane Study Group. *J Clin.Oncol.* 2000;**18**:1399-411.

90. Nabholtz JM, Buzdar A, Pollak M, Harwin W, Burton G, Mangalik A *et al.* Anastrozole is superior to tamoxifen as first-line therapy for advanced breast cancer in postmenopausal women: results of a North American multicenter randomized trial. Arimidex Study Group. *J Clin.Oncol.* 2000;**18**:3758-67.

91. Mouridsen H, Gershanovich M, Sun Y, Perez-Carrion R, Boni C, Monnier A *et al.* Superior efficacy of letrozole versus tamoxifen as first-line therapy for postmenopausal women with

advanced breast cancer: results of a phase III study of the International Letrozole Breast Cancer Group. *J Clin.Oncol.* 2001;**19**:2596-606.

92. Paridaens R, Dirix, L. Y., and Lorisch C. Promising activity and safety of exemestane (E) as first line hormonal therapy (HT) in metastatic breast cancer (MBC) patients (pts): final results of an EORTC randomised Phase II trial. Breast Cancer Res Treat. 2000.
Ref Type: Abstract

93. Thurlimann B, Keshaviah A, Coates AS, Mouridsen H, Mauriac L, Forbes JF *et al.* A comparison of letrozole and tamoxifen in postmenopausal women with early breast cancer. *N.Engl.J Med* 2005;**353**:2747-57.

94. Baum M, Buzdar A, Cuzick J, Forbes J, Houghton J, Howell A *et al.* Anastrozole alone or in combination with tamoxifen versus tamoxifen alone for adjuvant treatment of postmenopausal women with early-stage breast cancer: results of the ATAC (Arimidex, Tamoxifen Alone or in Combination) trial efficacy and safety update analyses. *Cancer* 2003;**98**:1802-10.

95. Coombes RC, Hall E, Gibson LJ, Paridaens R, Jassem J, Delozier T *et al.* A randomized trial of exemestane after two to three years of tamoxifen therapy in postmenopausal women with primary breast cancer. *N.Engl.J Med* 2004;**350**:1081-92.

96. Baum M, Budzar AU, Cuzick J, Forbes J, Houghton JH, Klijn JG *et al.* Anastrozole alone or in combination with tamoxifen versus tamoxifen alone for adjuvant treatment of postmenopausal women with early breast cancer: first results of the ATAC randomised trial. *Lancet* 2002;**359**:2131-9.

97. Goss PE, Ingle JN, Martino S, Robert NJ, Muss HB, Piccart MJ *et al.* A randomized trial of letrozole in postmenopausal women after five years of tamoxifen therapy for early-stage breast cancer. *N.Engl.J Med* 2003;**349**:1793-802.

98. Jakesz R, Jonat W, Gnant M, Mittlboeck M, Greil R, Tausch C *et al.* Switching of postmenopausal women with endocrine-responsive early breast cancer to anastrozole after 2 years' adjuvant tamoxifen: combined results of ABCSG trial 8 and ARNO 95 trial. *Lancet* 2005;**366**:455-62.

99. Cameron DA, Kerr G, Jack W, and Dixon JM. Aromatase Inhibitors Up Front: The Switch of 2-5 Years or Extended Adjuvant - How do we choose? Presentation at Nottingham International Breast Cancer Conference 2005 . 2005.
Ref Type: Abstract

100. Thomas R, Godward S, Makris A, Bloomfield D, Moody AM, Williams M. Giving patients a choice improves quality of life: a multi-centre, investigator-blind, randomised, crossover study comparing letrozole with anastrozole. *Clin Oncol (R.Coll.Radiol.)* 2004;**16**:485-91.

101. Dixon JM, Love CD, Bellamy CO, Cameron DA, Leonard RC, Smith H *et al.* Letrozole as primary medical therapy for locally advanced and large operable breast cancer. *Breast Cancer Res Treat.* 2001;**66**:191-9.

102. Santen RJ, Yue W, Naftolin F, Mor G, Berstein L. The potential of aromatase inhibitors in breast cancer prevention. *Endocr.Relat Cancer* 1999;**6**:235-43.

103. Goss PE. Risks versus benefits in the clinical application of aromatase inhibitors. *Endocr.Relat Cancer* 1999;**6**:325-32.

104. Dixon JM, Renshaw L, Bellamy C, Stuart M, Hoctin-Boes G, Miller WR. The effects of neoadjuvant anastrozole (Arimidex) on tumor volume in postmenopausal women with breast cancer: a randomized, double-blind, single-center study. *Clin.Cancer Res* 2000;**6**:2229-35.

105. Miller WR,.Dixon JM. Endocrine and clinical endpoints of exemestane as neoadjuvant therapy. *Cancer Control* 2002;**9**:9-15.

106. Goss PE,.Strasser K. Aromatase inhibitors in the treatment and prevention of breast cancer. *J Clin.Oncol.* 2001;**19**:881-94.

107. Jonat W, Howell A, Blomqvist C, Eiermann W, Winblad G, Tyrrell C *et al.* A randomised trial comparing two doses of the new selective aromatase inhibitor anastrozole (Arimidex) with megestrol acetate in postmenopausal patients with advanced breast cancer. *Eur.J Cancer* 1996;**32A**:404-12.

108. Buzdar A, Jonat W, Howell A, Jones SE, Blomqvist C, Vogel CL *et al.* Anastrozole, a potent and selective aromatase inhibitor, versus megestrol acetate in postmenopausal women with advanced breast cancer: results of overview analysis of two phase III trials. Arimidex Study Group. *J Clin.Oncol.* 1996;**14**:2000-11.

109. Gershanovich M, Chaudri HA, Campos D, Lurie H, Bonaventura A, Jeffrey M *et al.* Letrozole, a new oral aromatase inhibitor: randomised trial comparing 2.5 mg daily, 0.5 mg daily and aminoglutethimide in postmenopausal women with advanced breast cancer. Letrozole International Trial Group (AR/BC3). *Ann.Oncol.* 1998;**9**:639-45.

110. Goss PE, Winer EP, Tannock IF, Schwartz LH. Randomized phase III trial comparing the new potent and selective third-generation aromatase inhibitor vorozole with megestrol acetate in postmenopausal advanced breast cancer patients. North American Vorozole Study Group. *J Clin.Oncol.* 1999;**17**:52-63.

111. Hamilton A,.Piccart M. The third-generation non-steroidal aromatase inhibitors: a review of their clinical benefits in the second-line hormonal treatment of advanced breast cancer. *Ann.Oncol.* 1999;**10**:377-84.

112. Dixon, J. M. Neoadjuvant endocrine therapy. In "Breast Cancer: Diagnosis and Management". Dixon, J. M. 1, 305-309. 2000. The Netherlands, Elsevier Science.
Ref Type: Serial (Book,Monograph)

113. Smith IE, Dowsett M, Ebbs SR, Dixon JM, Skene A, Blohmer JU *et al.* Neoadjuvant treatment of postmenopausal breast cancer with anastrozole, tamoxifen, or both in combination: the Immediate Preoperative Anastrozole, Tamoxifen, or Combined with Tamoxifen (IMPACT) multicenter double-blind randomized trial. *J Clin Oncol* 2005;**23**:5108-16.

114. Cataliotti L, Buzdar AU, Noguchi S, Bines J, Takatsuka Y, Petrakova K *et al.* Comparison of anastrozole versus tamoxifen as preoperative therapy in postmenopausal women with hormone receptor-positive breast cancer: the Pre-Operative "Arimidex" Compared to Tamoxifen (PROACT) trial. *Cancer* 2006;**106**:2095-103.

115. Semiglazov VF, Semiglazov V, Ivanov V, et al. The relative efficacy of neoadjuvant endocrine therapy vs chemotherapy in postmenopausal women with ER positive breast cancer. *Proc Am Soc Clin Oncol* 2004;**22**.

116. Semiglazov VF and et al. Exemestane vs Tamoxifen as neoadjuvant endocrine therapy for postmenopausal women with ER positive breast cancer. ASCO Annual meeting poster presentation . 2005.
Ref Type: Abstract

117. Goldie JH. Scientific basis for adjuvant and primary (neoadjuvant) chemotherapy. *Semin.Oncol.* 1987;**14**:1-7.

118. Dixon JM, Jackson J, Renshaw L, Miller WR. Neoadjuvant tamoxifen and aromatase inhibitors: comparisons and clinical outcomes. *J Steroid Biochem.Mol.Biol.* 2003;**86**:295-9.

119. Osborne CK, Yochmowitz MG, Knight WA, III, McGuire WL. The value of estrogen and progesterone receptors in the treatment of breast cancer. *Cancer* 1980;**46**:2884-8.

120. Horwitz KB, Koseki Y, McGuire WL. Estrogen control of progesterone receptor in human breast cancer: role of estradiol and antiestrogen. *Endocrinology* 1978;**103**:1742-51.

121. Chang J, Powles TJ, Allred DC, Ashley SE, Makris A, Gregory RK *et al.* Prediction of clinical outcome from primary tamoxifen by expression of biologic markers in breast cancer patients. *Clin.Cancer Res* 2000;**6**:616-21.

122. Gerdes J, Schwab U, Lemke H, Stein H. Production of a mouse monoclonal antibody reactive with a human nuclear antigen associated with cell proliferation. *Int.J Cancer* 1983;**31**:13-20.

123. Bouzubar N, Walker KJ, Griffiths K, Ellis IO, Elston CW, Robertson JF *et al.* Ki67 immunostaining in primary breast cancer: pathological and clinical associations. *Br J Cancer* 1989;**59**:943-7.

124. Harper-Wynne C, Ross G, Sacks N, Salter J, Nasiri N, Iqbal J *et al.* Effects of the aromatase inhibitor letrozole on normal breast epithelial cell proliferation and metabolic indices in postmenopausal women: a pilot study for breast cancer prevention. *Cancer Epidemiol.Biomarkers Prev.* 2002;**11**:614-21.

125. Makris A, Powles TJ, Allred DC, Ashley S, Ormerod MG, Titley JC *et al.* Changes in hormone receptors and proliferation markers in tamoxifen treated breast cancer patients and the relationship with response. *Breast Cancer Res Treat.* 1998;**48**:11-20.

126. Olayioye MA, Neve RM, Lane HA, Hynes NE. The ErbB signaling network: receptor heterodimerization in development and cancer. *EMBO J* 2000;**19**:3159-67.

127. Klapper LN, Kirschbaum MH, Sela M, Yarden Y. Biochemical and clinical implications of the ErbB/HER signaling network of growth factor receptors. *Adv.Cancer Res* 2000;**77**:25-79.

128. Dixon JM, Jackson J Hills M Renshaw L Cameron DA Anderson TJ Miller WR Dowsett M. Anastrozole demonstrates clinical and biological effectiveness in erbB2 ER positive breast cancers. Vol 76,supp 1;Dec 02; pS75;abstract 263. Breast Cancer Research and Treatment. 76(supp 1), S75. 2002.
Ref Type: Abstract

129. Ellis MJ, Coop A, Singh B, Mauriac L, Llombert-Cussac A, Janicke F *et al.* Letrozole is more effective neoadjuvant endocrine therapy than tamoxifen for ErbB-1- and/or ErbB-2-positive, estrogen receptor-positive primary breast cancer: evidence from a phase III randomized trial. *J Clin.Oncol.* 2001;**19**:3808-16.

130. Dixon JM, Renshaw L, Bellany C. Efficacy of anastrozole as neoadjuvant therapy in postmenopausal women with large operable breast cancers: reductions in tumour volume. *Breast* 1999;215.

131. Dixon JM, Senbanjo RO, Anderson TJ, Forrest AP, Elton RA. Clinical assessment of tumour size in primary breast carcinoma. *Clin.Oncol.* 1984;**10**:117-21.

132. Warr D, McKinney S, Tannock I. Influence of measurement error on assessment of response to anticancer chemotherapy: proposal for new criteria of tumor response. *J Clin.Oncol.* 1984;**2**:1040-6.

133. Brindley CO, Markoff E, Schneiderman MA. Direct observation of lesion size and number as a method of following the growth of human tumors. *Cancer* 1959;**12**:139-46.

134. Cocconi G, Di Blasio B, Alberti G, Bisagni G, Botti E, Peracchia G. Problems in evaluating response of primary breast cancer to systemic therapy. *Breast Cancer Res Treat.* 1984;**4**:309-13.

135. Forouhi P, Walsh JS, Anderson TJ, Chetty U. Ultrasonography as a method of measuring breast tumour size and monitoring response to primary systemic treatment. *Br J Surg* 1994;**81**:223-5.

136. Elston CW,.Ellis IO. Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. C. W. Elston & I. O. Ellis. Histopathology 1991; 19; 403-410. *Histopathology* 2002;**41**:151.

137. Harvey JM, Clark GM, Osborne CK, Allred DC. Estrogen receptor status by immunohistochemistry is superior to the ligand-binding assay for predicting response to adjuvant endocrine therapy in breast cancer. *J Clin.Oncol.* 1999;**17**:1474-81.

138. Simpson JF, Dutt PL, Page DL. Expression of mitoses per thousand cells and cell density in breast carcinomas: a proposal. *Hum.Pathol.* 1992;**23**:608-11.

139. Johnston SR, Boeddinghaus IM, Riddler S, Haynes BP, Hardcastle IR, Rowlands M *et al.* Idoxifene antagonizes estradiol-dependent MCF-7 breast cancer xenograft growth through sustained induction of apoptosis. *Cancer Res* 1999;**59**:3646-51.

140. Bonadonna G, Valagussa P, Brambilla C, Moliterni A, Zambetti M, Ferrari L. Adjuvant and neoadjuvant treatment of breast cancer with chemotherapy and/or endocrine therapy. *Semin.Oncol.* 1991;**18**:515-24.

141. Dowsett M,.Ellis MJ. Role of biologic markers in patient selection and application to disease prevention. *Am.J Clin Oncol.* 2003;**26**:S34-S39.

142. Jackson J, Miller WR, Dixon JM. Safety issues surrounding the use of aromatase inhibitors in breast cancer. *Expert.Opin.Drug Saf* 2003;**2**:73-86.

143. Archer CD, Parton M, Smith IE, Ellis PA, Salter J, Ashley S *et al.* Early changes in apoptosis and proliferation following primary chemotherapy for breast cancer. *Br J Cancer* 2003;**89**:1035-41.

144. Bottini A, Berruti A, Bersiga A, Brizzi MP, Bruzzi P, Aguggini S *et al.* Relationship between tumour shrinkage and reduction in Ki67 expression after primary chemotherapy in human breast cancer. *Br J Cancer* 2001;**85**:1106-12.

145. Geisler J, Detre S, Berntsen H, Ottestad L, Lindtjorn B, Dowsett M *et al.* Influence of neoadjuvant anastrozole (Arimidex) on intratumoral estrogen levels and proliferation markers in patients with locally advanced breast cancer. *Clin Cancer Res* 2001;**7**:1230-6.

146. Urruticoechea A, Smith IE, Dowsett M. Proliferation marker Ki-67 in early breast cancer. *J Clin Oncol* 2005;**23**:7212-20.

147. Miller WR, Dixon JM, Cameron DA, Anderson TJ. Biological and clinical effects of aromatase inhibitors in neoadjuvant therapy. *J Steroid Biochem.Mol.Biol.* 2001;**79**:103-7.

148. Anderson TJ, Dixon JM, Stuart M, Sahmoud T, Miller WR. Effect of neoadjuvant treatment with anastrozole on tumour histology in postmenopausal women with large operable breast cancer. *Br J Cancer* 2002;**87**:334-8.

149. Miller WR, Dixon JM, Macfarlane L, Cameron D, Anderson TJ. Pathological features of breast cancer response following neoadjuvant treatment with either letrozole or tamoxifen. *Eur.J Cancer* 2003;**39**:462-8.

150. Miller WR, Anderson TJ, Evans DB, Krause A, Hampton G, Dixon JM. An integrated view of aromatase and its inhibition. *J Steroid Biochem.Mol.Biol.* 2003;**86**:413-21.

151. Clark GM,.McGuire WL. Steroid receptors and other prognostic factors in primary breast cancer. *Semin.Oncol* 1988;**15**:20-5.

152. Bajetta E, Zilembo N, Bichisao E. Aromatase inhibitors in the treatment of postmenopausal breast cancer. *Drugs Aging* 1999;**15**:271-83.

153. Harper-Wynne CL, Sacks NP, Shenton K, MacNeill FA, Sauven P, Laidlaw IJ *et al.* Comparison of the systemic and intratumoral effects of tamoxifen and the aromatase inhibitor vorozole in postmenopausal patients with primary breast cancer. *J Clin Oncol* 2002;**20**:1026-35.

154. Dixon JM, Love CD, Renshaw L, Bellamy C, Cameron DA, Miller WR *et al.* Lessons from the use of aromatase inhibitors in the neoadjuvant setting. *Endocr.Relat Cancer* 1999;**6**:227-30.

155. Moneer M, El Didi M, Khaled H. Breast conservative surgery: is it appropriate for locally advanced breast cancer following downstaging by neoadjuvant chemotherapy? A pathological assessment. *Breast* 1999;**8**:315-9.

156. Veronesi U, Bonadonna G, Zurrida S, Galimberti V, Greco M, Brambilla C *et al.* Conservation surgery after primary chemotherapy in large carcinomas of the breast. *Ann.Surg* 1995;**222**:612-8.

157. Makris A, Powles TJ, Ashley SE, Chang J, Hickish T, Tidy VA *et al.* A reduction in the requirements for mastectomy in a randomized trial of neoadjuvant chemoendocrine therapy in primary breast cancer. *Ann.Oncol* 1998;**9**:1179-84.

158. Cameron, D. A., Jack W, Forouhi P, Keen J, Dixon, J. M., Leonard, R. C., and Chetty U. Oestrogen receptor directed primary systemic therapy: a randomised trial compared with conventional therapy in operable breast cancer. Breast Cancer Res Treat. 76 (suppl)(S5). 2002. Ref Type: Abstract

Section 8: Appendices

Appendix 1: Letrozole Audit Patient information sheet

LETROZOLE AUDIT
EDINBURGH BREAST UNIT
INFORMATION FOR PATIENTS

We would like to invite you to take part in a medical audit looking at the drug Letrozole. The audit will be conducted here at Edinburgh Breast Unit, by the surgeons and medical oncologists. This audit has been approved by a research ethics committee.

Introduction / Background

Approximately two thirds of all breast cancers, in women whose periods have stopped, need the female hormone oestrogen to grow, therefore one way of treating breast cancer is to use drugs which will deprive the cancer of oestrogen. Letrozole works by stopping the body producing oestrogen and deprives the breast cancer of the oestrogen it requires.

What are the options for your treatment?

Your doctor has told you that you have breast cancer and that there are different treatments available. He / she has decided however, that the best treatment for you would be hormonal therapy in the first instance. This will mean you take drugs either before you have breast surgery or it may be that you continue to take hormonal drugs without having an operation to your breast. The majority of women who have hormone sensitive tumours over 2cm in size can be made smaller using this therapy so if you do need an operation, then the amount of surgery which will follow the treatment is likely to be less extensive than would have been required without this treatment.

Purpose of the audit

Although the majority of tumours can be made smaller using hormone therapy, we do not yet have a test which will allow us to tell within a few

weeks of starting treatment whether it will be effective. At the present time therefore, we give all patients three months of treatment and during this time we carefully measure the size of the tumour monthly using clinical measurements and ultrasound. During this study we would like to take a sample of your breast cancer 14 days after you start Letrozole treatment. Only a very small amount of tissue is needed and this can be removed with a special automatic biopsy needle. The needle test is performed after the breast and skin itself is numbed by injecting local anaesthetic.
It is then hoped you will continue taking hormonal treatment for 3 months. At the end of 3 months you will either have another core biopsy or have an operation.
By comparing the changes in the biopsy 10-14 days after treatment and after 3 months of treatment with the initial sample obtained at diagnosis. It should be possible for us to look at the changes in your tumour over the whole of the 3 months treatment course.

Description of drug treatment

If you agree to take part in this audit, you will be given Letrozole 2.5mgs to take once a day for 3 months in the first instance.

Participation in this audit

Participation is entirely voluntary and you are free to withdraw at any time if you wish to do so. If you refuse participation or withdraw from the audit, your medical care will not be affected in any way. Your doctor may discontinue your participation in the audit if he/she judges it to be in your best interest. Your doctor will inform you of any significant new findings related to Letrozole, which may relate to your participation in this audit. When you consent, your GP will be told that you are taking part in this audit. You should not have any additional costs as a result of taking part in this audit because all the extra tests will be performed during standard hospital visits.

Procedures during the three months treatment

DIAGNOSTIC CORE BIOPSY
(POSITIVE FOR OESTROGEN)
RECEPTORS

ULTRASOUND
CLINICAL ASSESSMENT
COMMENCE LETROZOLE 2.5mgs DAILY

↓

10-14 DAY CORE BIOPSY
(Optional)

↓

SIX WEEKS

ULTRASOUND
CLINICAL ASSESSMENT
DRUG TOLERABILITY

↓

MONTH THREE

ULTRASOUND
CLINICAL ASSESSMENT
MAMMOGRAM
DRUG TOLERABILITY
FINAL SURGERY or
CONTINUE TAKING LETROZOLE or TAMOXIFEN

↓

AUDIT COMPLETE

Possible benefits

This treatment should reduce the size of your tumour which if you do have an operation, should allow you to have less extensive surgery. If you do not

proceed to surgery then the tablets will keep your breast cancer under control. This audit will allow us to determine whether your tumour does respond to Letrozole, and will help your doctor treat your cancer more effectively.

Potential side effects of Letrozole

In trials in which patients were treated with Letrozole for breast cancer, side effects were generally mild to moderate and rarely severe enough to require stopping treatment.
The more common side effects that have been seen in patients treated with Letrozole 2.5mgs are:
Tiredness, hot flushes or increased sweating, changes in weight, dizziness, itching and skin rash, headache, changes in appetite, water retention, nausea and sickness, constipation or diarrhoea, stomach upset or pain. Less common side effects are chest pain, viral infections, pain in the muscles, bones and joints, shortness of breath and coughing.

DO NOT BE ALARMED BY THIS LIST OF SIDE EFFECTS. YOU MAY NOT HAVE ANY OF THEM.

YOU SHOULD REPORT ANY DISCOMFORT OR PROBLEM THAT YOU ARE CONCERNED ABOUT TO YOUR DOCTOR IMMEDIATELY.

Contact numbers:

Mr J.M.Dixon
Consultant Surgeon
Edinburgh Breast Unit
Western General Hospital
Tel: 0131 537 2643 (office)

Lorna Renshaw
Research Nurse
Edinburgh Breast Unit
Western General Hospital
Tel: 0131 537 1615 or 0131 537 1000 ask for bleep 8559

Miss Juliette Murray
Research Fellow
Edinburgh Breast Unit
Western General Hospital
Tel: 0131 537 2907

Appendix 2: Patient information sheet and consent form for core biopsy

Edinburgh Breast Unit
Surgical and Associated Services Division
Western General Hospital, Crewe Road, Edinburgh, EH4 2XU
Consultants: Miss EDC Anderson, Mr U Chetty, Mr JM Dixon, Mr GT Neades, Mr RJ Salem
Unit Co-ordinator: Mrs S Watchman
General Office: 0131 537 1611 Ward 6: 0131 537 1631 Fax No: 0131 537 1004

Patient Information

Studies on Breast Disease

You are about to have a core biopsy performed to determine the cause of your breast lump. Following injection of local anaesthetic, thin slivers of tissue obtained by a biopsy needle will be taken and sent to the pathology department to diagnose the cause of your breast lump.

The Edinburgh Breast Unit is involved in a number of research projects looking at the effect of drugs on breast lumps. We write to invite you to allow us to take extra slivers of tissue from your lump which might be used in future research should you receive any drug treatment.

If a definite diagnosis is not obtained from the specimens we send to the pathology department, then the stored samples will be made available to the pathology department to help diagnose the cause of your breast lump.

Edinburgh Breast Unit
Surgical and Associated Services Division
Western General Hospital, Crewe Road, Edinburgh, EH4 2XU
Consultants: Miss EDC Anderson, Mr U Chetty, Mr JM Dixon, Mr GT Neades, Mr RJ Salem
Unit Co-ordinator: Mrs S Watchman
General Office: 0131 537 1611 Ward 6: 0131 537 1631 Fax No: 0131 537 1004

Patient Consent Form

Studies on Breast Disease

Please Initial Boxes

I have read the attached information sheet. ☐

I agree to undergo the procedure of core biopsy. I understand that the purpose of the core biopsy is to determine the cause of my breast lump. ☐

I agree to allow extra samples of my breast lump to be taken. I understand how the sample will be collected and that giving this sample is voluntary and that I am free to withdraw my approval for use of the sample at any time without giving a reason and without my medical treatment or legal rights being affected. ☐

I understand that the research performed on the extra sample may include research to improve our understanding of how drugs influence breast disease. ☐

I understand that if these extra samples are used for research, ethical approval will be obtained for the research project and that I will be asked to sign a separate consent form giving permission so these extra samples can be used. ☐

I have read and understood the consent form.

I consent to having a core needle biopsy.

Signature of Patient .....

Name of Patient .....

Date .....

Consent Witnessed by ..... Page 2 of 2
Generic Consent Form120302

Appendix 3: Determination of Histological Grade

Tissue used for grading was fixed in 10% neutral buffered formalin and 4μm sections were stained with haematoxylin and eosin. Grading was carried out on invasive cancer only using the modified criteria of Bloom and Richardson. It was assessed as follows,

Tubule formation

Care was taken to differentiate between clefts due to tumour tissue shrinkage and tubule formation.

Score 1 – great majority of tumour composed of tubules with clearly visible lumina

2 – moderate amount of tubule formation but with areas of solid tumour growth

3 – little or no tubule formation, the cells mainly growing in sheets or cords.

Nuclear pleomorphism

The variability of both size and shape of tumour nuclei was assessed and scored.

Score 1 – little variation in size and shape of nuclei

2 – moderate variation without extremes of cell size or shape

3 – marked variation present with large bizarre nuclei and multiple nucleoli.

Mitotic Rate

Using a magnification of x 300 the number of mitoses per 10 fields were counted.

Score 1 – <10 mitoses per 10 fields

2 – 10-19 mitoses per 10 fields

3 – ≥ 20 mitoses per 10 fields

To obtain the overall tumour grade the scores for each category were added, giving a possible score of between 3 and 9 points. Grade was then allocated on the following basis;

Grade 1 – well differentiated – 3-5 points

Grade 2 – moderately differentiated – 6-7 points

Grade 3 – poorly differentiated – 8-9 points.

Appendix 4: Preparation of blocks (Specimen Automated Processing Protocol)

Tissue was fixed in 4% buffered formalin for 24 hours before processing. The tissue was then placed in a cassette and processed on a Vacuum Infiltrated Processor (VIP, Tissue Tech VIP, E300 series, Miles Inc) overnight using the following protocol;

- Alcohol 50%, 2 hours at 35°C
- Alcohol 80%, 1 hour at 35°C
- Alcohol 95%, 1 hour at 35°C
- Alcohol 100%, 1 hour at 35°C
- Alcohol 100%, 1 hour at 35°C
- Alcohol 100%, 2 hours at 35°C
- Xylene 100%, 3 changes of 1 hour each
- Paraffin, 4 changes of 1 hour each at 60°C

The tissue was then placed in mould and covered in paraffin wax.

Appendix 5: Haematoxylin and Eosin staining methodology

Materials

Harris Haematoxylin (6765004)	SHANDON
Eosin (CI 453380)	SHANDON
Pertex mounting resin	CELL PATH

Method

1) Dewax and rehydrate sections through graded alcohols to water
2) Immerse in haematoxylin (0.4%) for 30 seconds
3) Wash sections through 3 changes of tap water 1 minute each
4) Dip in acid alcohol (70% alcohol + 1% HCl) 4-5 times
5) Rinse in water
6) Immerse in Scott's tap water for 30 seconds to blue
7) Immerse in eosin (0.5% eosin Y solution) for 15 seconds
8) Wash sections in tap water for 1 minute
9) Dehydrate sections through graded alcohols to absolute alcohol (2 changes)
10) Sections through 3 changes of xylene and mount with pertex

Nuclear staining appears blue/purple. Cytoplasm, collagen, keratin and erythrocytes stain pink.

Appendix 6: En-Vision Immunohistochemistry Protocol

Materials

DAKO EnVision kit	DAKOCYTOMATION
Serum Free protein block	DAKOCYTOMATION
Avidin/Biotin blocking kit	VECTOR Laboratories
Tris	SIGMA
NaCl	SIGMA
HCl	SIGMA
EDTA	SIGMA
Citric Acid	SIGMA
tri-sodium citrate	SIGMA
cupric sulphate ($CuSO_{4)}$	SIGMA
DAKO envision kit (K5007)	DAKOCYTOMATION
Hydrogen peroxide H_2O_2 (H1009)	SIGMA
Avidin / Biotin Block (Sp2001)	VECTOR
Dako Protein Block (X0909)	DAKOCYTOMATION
DAB & Substrate (K3468)	DAKOCYTOMATION

Desired primary antibody:

Ki-67	clone MIB-1. Batch number - 012.	DAKOCYTOMATION

ER-a	clone 6F11.	NOVACASTRA
	(From mouse melanoma p3-NS1-Ag4-1)	
PgR	clone PgR636. Batch or lot number 110.	DAKOCYTOMATION

Solutions

Tri Buffered Saline (TBS)

Solution 1 (Tris/HCl)

- Add 30.25g Tris in dH_2O + 200ml NHCl (85ml/L H_2O)
- Make up to 2L dH_2O

Solution 2 (Normal Saline)

- Dissolve 8.5g NaCl in 1L dH_2O

TBS Working Solution

- Add 100ml Tris/HCl to 900ml normal saline

Tris-EDTA Buffer (0.05M/ 50x Conc)

- Dissolve 1.85g EDTA + 2.75g Tris in 500ml dH_2O
- May take a few hours to dissolve completely

Working solution (0.001M)

- Add 30ml 50x solution to 1470ml dH_2O

Citrate Buffer (pH6 50x conc/ 0.5M)

- Dissolve 7.5g citric acid + 60.3g tri-sodium citrate ($2H_2O$) in 500ml dH_2O

Working solution (0.01M)

- Add 30ml 50x solution to 1470ml dH_2O

3% H_2O_2

- Add 45ml H_2O_2 stock (30%) to 405ml dH_2O

Copper DAB Enhancement Solution

- Add 3g cupric sulphate ($CuSO_4$) + 5.35g NaCl to 450ml dH_2O

Method

Preparation

- Prepare antigen retrieval buffer (citrate and EDTA), 3% H_2O_2 and TBS
- Bring antigen retrieval buffer up to pressure in the pressure cooker in the microwave – 15 min at full power then 40% power until required
- Dewax sections in xylene for 5-10 min to allow all paraffin wax to dissolve
- Bring down through the alcohols to H_2O and rinse for 1-2min
- Place in 3% H_2O_2 for 5 mins to block endogenous peroxidase activity then rinse in H_2O

Antigen Retrieval

- Place sections in antigen retrieval buffer and microwave for 6min at full power (should reach full pressure for at least 2 min)
- Run cold H_2O into hot antigen retrieval buffer in pressure cooker. When cool place sections in water and rinse for 1-2 min
- Put sections in sequenza trays and wash with TBS

Blocking

- Block endogenous peroxidase by adding 2-3 drops of protein block (DAKO: X0909) to each section. Incubate for 5min.
- Wash with TBS

Primary Antibody

- Add 100µl primary antibody at relevant concentration in antibody diluent (DAKO:S2022) for desired time
- Wash with TBS

En-Vision

- Add 100µl En-Vision chemmate solution (DAKO kit:K5007) for 30 min
- Wash with TBS

Chromogen

- Add 100µl DAB solution (DAKO ki: K5007) and incubate in the dark for 10min
- Wash with dH_2O

- Place sections in a slide tray and rinse in H_2O for 1-2min
- Incubate for 5 min in $CuSO_4$ to enhance DAB staining
- Rinse in H_2O

Counterstain

- Counterstain in haematoxylin for 30secs and wash in H_2O
- Dip sections in Scott's Tap Water for a few seconds until blue
- Rinse in H_2O
- Take up through graded alcohols to absolute alcohol and mount slides from xylene then coverslip

Positively stained structures appear brown when viewed under direct light microscopy

Primary antibody concentrations and incubation periods

antigen	retrieval	primary antibody conc	Incubation time
ER-a	EDTA	1 in 25	1hr
PgR	Citrate	1 in 100	30 min
Ki-67	Citrate	1 in 50	30 min

Appendix 7: Allred scoring of ER/PgR stained slides - results of blind scoring of the same sections by two investigators at different time points

CASE	scorer 1		scorer 2	
NO	Proportion	Intensity	Proportion	Intensity
1	5	3	5	2
2	5	2	5	2
3	5	3	5	3
4	5	2	5	2
5	5	3	5	3
6	1	2	2	1
7	5	3	5	3
8	5	3	5	3
9	5	2	5	3
10	5	3	5	2
11	5	2	5	2
12	5	2	5	2
13	5	2	5	2
14	5	3	5	3
15	4	2	4	1
16	5	3	5	3
17	5	3	5	3
18	5	3	5	2
19	5	3	5	3
20	5	3	5	3
21	5	2	5	2
22	5	3	5	3
23	3	2	3	2
24	5	2	5	2
25	1	1	1	2
26	5	2	5	3
27	5	2	5	2
28	5	3	5	3
29	5	3	5	2
30	5	3	5	3
31	5	3	5	3
32	5	2	5	2
33	5	3	5	3
34	5	2	5	3
35	5	3	5	2
36	5	3	5	3
37	5	3	5	2
38	5	2	5	2
39	5	2	5	2
40	5	3	5	3

41	1	1	1	1
42	5	2	5	2
43	5	3	5	2
44	5	2	5	2
45	5	3	5	3
46	5	2	5	2
47	4	2	4	2
48	5	3	5	3
49	5	2	5	2
50	5	2	5	2

Appendix 8: Allred scoring of ER/PgR stained slides - results of blind scoring of the same sections by one investigator at two different time points

CASE	score 1	dec	score 2	May
NO	Proportion	Intensity	Proportion	Intensity
1	5	3	5	3
2	4	2	4	2
3	5	2	5	2
4	5	2	5	2
5	3	1	2	1
6	5	3	5	3
7	5	2	5	2
8	5	3	5	3
9	5	2	5	2
10	5	2	5	2
11	5	2	5	2
12	5	2	5	2
13	5	3	5	3
14	5	2	5	2
15	5	3	5	2
16	5	1	5	1
17	5	2	5	2
18	4	2	4	2
19	5	2	5	2
20	5	2	5	2

Appendix 9: Assessment of proliferation, results of blind scoring of the same sections by two investigators

Re-scored cases

Case	Pre	10-14d	3m
58 - count1	24.06	2.67	0.84
58 - count 2	19.34	3.46	2.03
difference	4.72	0.79	1.19
% variation	21.75		82.92
66 - count1	5.71	3.25	1.09
66 - count2	7.81	3.54	1.76
difference	2.1	0.31	0.67
% variation	31.65	9.13	47.01
51 - count 1	15.53	13.4	16.8
51 - count2	15.73		18.18
difference	0.2		1.38
% variation	1.31		7.89
2 - count 1	19.16	11.12	7.07
2 - count2	23.44	10.85	8.96
difference	4.28	0.27	1.89
% variation	20.09	2.45	23.58
10 - count 1	9.83	2.4	0.08
10 - count 2	14.71	2.02	0.73
difference	4.88	0.38	0.65
% variation	39.77	31.4	156.09

Standard dev	42.07772
SEM	11.25073

Appendix 10: Assessment of proliferation, results of blind scoring of the same sections by one investigator on two separate occasions

Case	count 1	count 2
Let 58a	24.06	19.34
Let 58b	2.67	3.46
Let 58c	0.84	2.03
Let 66a	5.71	7.81
Let 66b	3.25	3.54
Let 66c	1.09	1.76
Let 51a	15.53	15.73
Let 51b	16.8	18.18
Let 2a	19.16	23.44
Let 2b	11.12	10.85
Let 2c	7.07	8.96
Let 10a	9.83	14.71
Let 10b	2.4	2.02
Let 10c	0.08	0.73
Fem 10a	23.56	21.39
Fem 14a	26.92	21.92
Fem 17a	13.82	15.64
Fem 18b	11.22	13.02
Fem 35b	1.34	1.02
Fem 86b	1.21	2.16
Fem 15a	5.76	12.32

Pearson's correlation r = 0.9498

95% CI = 0.8782 – 0.9798, p= <0.0001

Appendix 11: HercepTest immunohistochemistry for HER 2 (erbB2)

Materials:

Antigen retrieval solution	30ml Epitope retrieval solution [DAKO X10 conc code K5205 lot 023(101)]in 270mls distilled water
Wash buffer (10 x concentrated)	DAKO code K5205 lot 494
Control slides code	K5205 lot 023(201):

(Each slide contains sections of three formalin fixed paraffin embedded breast carcinoma cell lines representing different levels of HER2 protein expression: MDA-231 (0), MDA – 175 (1+) and SK-BR-3 (3+)

DAKO HercepTest for Autostainer (for immunocytochemical determination of HER2 protein over expression in fixed, paraffin-embedded breast carcimonas) Contains:

- 45ml of Peroxidase blocking reagent : 3% hydrogen peroxidase containing 15 mmol/L NaN3
- 24ml Rabbit Anti-Human HER2 Protein: Antibody containing 15 mmol/L NaN3
- 45ml Visualisation Reagent: Dextran polymer conjugated with horseradish peroxidase and goat anti-rabbit immunoglobulins

- 24ml Negative control reagent Immunoglobulin fraction of normal rabbit serum containing 15 mmol/L NaN3
- 50ml DAB buffered substrate substrate buffer solution containing hydrogen peroxide
- 2ml DAB Chromogen 3,3' Diaminobenzidine chromogen solution
- DAB solution was made by using 10ml of dab substrate and 10 drops of dab chromagen

Method

- Dewax paraffin embedded sections in xylene for 10 mins then take them down through gradient alcohols (100% x3 then 90%, 80%, 70%) to distilled water.
- Heat a water bath to 97^0C and fill baths with antigen retrieval solution (30ml Epitope retrieval solution [DAKO X10 conc code K5205 lot 023(101)]in 270mls distilled water).
- Put slides in antigen retrieval solution at 97^0C for 45 minutes.
- Allow solution to cool to room temperature over 30 mins.
- Load the slides onto a sequenza.
- Wash with TBS.
- Incubate sections in peroxidase blocking reagent for 5 mins.
- Rinse with distilled water and then with wash buffer.
- Incubate in primary antibody for 40 minutes.

- Rinsed with wash buffer
- Incubate in the visualisation solution for 30 minutes.
- Rinse with wash buffer.
- Apply DAB solution to the sections for 10 minutes
- Rinse with distilled water.
- Remove the sections from the sequenza
- Counterstain with haematoxylin for 1 minute
- Place for 5 minutes in tap water.
- Take the slides back up through gradient alcohol solutions to xylene before cover plating

Appendix 12: Method for fluorescence in situ hybridization (FISH) of 2+ sections on immunohistochemistry using DAKO Herceptest

This technique uses Pathvysion HER-2 neu kit for use on paraffin embedded material

- Cut 4μm sections and pick up on charged slides.
- Air dry slides overnight at 37°C and bake the following morning at 56°C for 2 hours
- Dewax sections in xylene for 10 mins
- Rinse slides in absolute ethanol x 2 and then air dry
- Immerse slides in 0.2M HCL for 25 mins
- Rinse slides in distilled water and then immerse in 2 x SSC wash buffer ph7.0 for 3 mins
- Drain off buffer and then immerse slides in pre-treatment solution at 80°C for 40 mins
- Remove slides from pre-treatment solution and then immerse in distilled water for one minute followed by 2x wash buffer for 3 mins
- Transfer slides to fresh pre-warmed wash buffer at 37°C for 15 mins
- Drain off excess buffer and then immerse slides in pre-heated protease solution plus enzyme @ 37°C for twenty minutes (digestion times vary depending on the material. Only add the protease enzyme to warmed protease buffer 4 minutes before use)
- Terminate digestion in wash buffer two times 5 mins
- Drain off excess buffer and then immerse slides in denature solution at 72°C for five minutes

- Transfer slides immediately to 70%, 80% and then 100% ethanol
- Air dry
- Warm probe to room temperature, vortex, centrifuge and vortex once more
- Add 10 microlitres of probe to section and then coverslip
- Seal coverslip with rubber cement and then place in a dark incubating chamber at 37°C overnight
- Next day soak off coverslips in post hybrid wash buffer in the dark at room temperature
- At the same time pre-warm post hybrid wash solution to 72°C in the water bath
- Once coverslips are removed place slides in heated buffer for two minutes
- Remove slides from buffer and allow the slides to air dry upright in the dark
- Apply 10 microlitres of DAPI counterstain to the section and recover with a fresh coverslip
- Keep slides in the dark until ready for viewing

Appendix 13: Licensed indications for Letrozole (as of August 2001)

Letrozole is licensed for:

- Treatment of early invasive breast cancer in postmenopausal women who have received prior standard adjuvant tamoxifen therapy

- First line treatment in postmenopausal women with breast cancer

- Treatment of advanced breast cancer in postmenopausal women in whom tamoxifen or other anti-oestrogen has failed

- Pre-operative therapy in postmenopausal women with localised hormone receptor positive breast cancer, to allow subsequent breast-conserving surgery in women not originally considered candidates for breast-conserving surgery. Subsequent treatment after surgery should be in accordance with standard of care.

Appendix 14: Clinical, USS and Mammographic responses (measured at 3 mth)

Pt ID	Bidimentional % reduction / response USS	Tridimention-al % reduction / response USS	Bidimentio-nal % reduction / response Calliper	Tridimenti-onal % reduction / response Calliper	Bidimentio-nal % reduction / response Mammo	Tridimen-tional % reduction / response Mammo
01	↓13% NC	↓21% NC	↓58% NC	↓73% PR	Not able to assesss – died before 3 month assessment	----------
02	↓64% PR	↓71% PR	↓68% PR	↓82% PR	↓64% PR	↓78% PR
03	↓78% PR	↓92% PR	↓100% CR	↓100% CR	↓86% PR	↓94% PR
04	↓70% PR	↓79% PR	↓48% PR	↓64% PR	↓48% PR	↓64%PR
05	↑155% PD ↓75% PR	↓29% MD ↓79% PR	↑7% NC ↓24% NC	↑12% NC ↓34% MR	13 o'clock not visualised on mammo ↓63% PR	13 o'clock not visualised on mammo ↓76% PR
06	↓48% MR	↓78% PR	↓51% PR	↓66% PR	↓76% PR	↓88% PR
07	↓84% PR	↓91% PR	↓100% CR	↓100% CR	↓81% PR	91% PR
08	↓42% MR Medial tumour (multifocal ca)	↓36% MR Medial tumour (multifocal ca)	↓75% PR (Collective mass)	↓88% PR (Collective mass)	↓79% PR (Collective mass)	↓90% PR (Collectiv e mass)
09	NA	NA	NA	NA	NA	NA
10	↓77% PR	↓89% PR	↓100% CR	↓100% CR	↓74% PR	↓87% PR
11	↓28% MR	↓64% PR	↑2% NC	↑3% NC	↓27% MR	↓35% MR
12	↓30% MR	↓30% MR	↓14% NC	19.6 NC	Mammos not technically possible	Mammos not possible
13	↓46% MR	↓57% PR	↓39% MR	↓51% PR	↓41% MR	↓78% PR
14	↓61% PR	↓64% PR	↓78% PR	↓90% PR	↓51% PR	↓66% PR
15	↓91% PR	↓96% PR	↓87% PR	↓95% PR	↓85% PR	↓94% PR
16	↓56% PR	↓68% PR	↓100% CR	↓100% CR	↓87% PR	↓95% PR
17	↓69% PR	↓76% PR	↓66% PR	↓80% PR	↓49% MR	↓60% PR
18	↑ 7% NC ↓46% MR	↓13% NC ↓63% PR	↓60% PR ↓71% PR	↓74% PR ↓84% PR	↓65% PR Not measurable	↓80% PR Not measur
19	↓92% PR	↓97% PR	↓100% PR	↓100% PR	↓30% MR	↓40% MR
20	↓61% PR	↓68% PR	↓64% PR	↓79% PR	Mammos not done as patient found too painful	NA
21	↓37% MR	↓68% PR	↓100% CR	↓100% CR	↓100% CR	↓100% CR
22	↓47% MR	↓70% PR	↓68% PR	↓82% PR	↓20% NC	↓32% MR
23	↓54% PR	↓54% PR	↓50% PR	↓64% PR	↓57% PR	↓72% PR
24	↓79% PR	↓84% PR	↓77% PR	↓89% PR	↓70% PR	↓83% PR
25	↓84% PR	↓90%PR	↓60% PR	↓75% PR	↓30% MR	↓42% MR
26	↓49% MR	↓63% PR	↓79% PR	↓90% PR	↓57% PR	↓71% PR
27	↓26% MR	↓12% NC	↓19% NC	↓29% MR	↓10% NC	↓16% NC

Pt ID	Bidimentional % reduction / response USS	Tridimention-al % reduction / response USS	Bidimentio-nal % reduction / response Calliper	Tridimenti-onal % reduction / response Calliper	Bidimentio-nal % reduction / response Mammo	Tridimen-tional % reduction / response Mammo
28	↑41% PD	↑34% PD	↓0% NC	↓0% NC	↓9% NC	↓2% NC
29	↓92% PR	↓96% PR	↓91% PR	↓97% PR	↓43% MR	↓59% PR
30	↓76% PR	↓88% PR	↓100% CR	↓100% CR	↓77% PR	↓89% PR
31	↓67% PR	↓69% PR	↓56% PR	↓67% PR	↓67% PR	↓79% PR
32	58% PR	↓51% PR	↓65% PR	↓85% PR	↓58% PR	↓73% PR
33	Node - ↑33% PD	Node - ↑33% PD	Mass - ↓ 65% PR Node - ↓41% MR	Mass - ↓80% PR Node - ↓57% PR	Mass ↑ 2%	Mass ↓0%
34	↓90% PR	↓98% PR	↓81% PR	↓93% PR	Tumour not captured on mammo	Tumour not captured
35	↓41% MR ↓78% PR	↓56% PR ↓87% PR	↓100% PR ↓100% PR	↓100% PR ↓100% PR	↓38% MR ↓7% NC	↓52% PR ↓11% NC
36	↓67% PR ↓0% NC	↓66% PR ↓0% NC	↓57% PR Unable to assess – nil palpable pre/post treatment	↓68% PR Unable to assess – nil palpable pre/post treatment	↓41% MR ↓31% MR	↑34% MR ↓36% MR
37	Withdrew from study - poor health	----------	----------	----------	----------	----------
38	↓35% MR	↓49%MR	↓45% MR	↓59% PR	↓67% PR	↓81% PR
39	↓70% PR	↓81% PR	↓50% PR	↓65% PR	↓87% PR	↓95% PR
40	↑8% NC	↓37% MR	↓53%PR	↓13% NC	↓44% MR	↓59% PR
41	↓52% MR	↓50% MR	↓28% MR	↓50% MR	↓55% PR	↓70% PR
42	Withdrew side effects	NA	NA	NA	NA	NA
43	↓25% MR	↓35% MR	↓23% MR	↓32% MR	↓36% MR	↓47% MR
44	↓85% PR	↓95% PR	↓72% PR	↓86% PR	↓67% PR	↓80% PR
45	↓8% NC	↓48% MR	↓54% PR	↓69% PR	↓23% NC	↓36% MR
46	↓43% MR ↓40% MR	↓79% PR ↓69% PR	Nil palpable ↓7% NC	Nil palpable ↓72% PR	↓40% MR ↓42% MR	↓54% PR ↓56% MR
47	↓93% PR	↓96% PR	↓84% PR	↓94% PR	↓86% PR	↓95% PR
48	↓60% PR	↓70% PR	↓57% PR	↓66% PR	↓66% PR	↓82% PR
49	↓55% PR	↓66% PR	NA	NA	↓40% MR	↓53% PR
50	↓51% PR	↓60% PR	↓44% MR	↓58% PR	Tumour not measurable on mammo	Tumour not measurab
51	↓86% PR	↓94% PR	↓100% CR	↓100% CR	↓63% PR	↓77% PR
52	↓50% PR	↓57% PR	↓48% MR	↓61% PR	↓47% MR	↓61% PR
53	↓69% PR	↓79% PR	↓10% NC	↓14% NC	↓61% PR	↓75% PR
54	NA	NA	NA	NA	NA	NA
55	↓42% MR	↓42% MR	↓48% MR	↓64% PR		
56	↓46% MR	↓61% PR	↓90% PR	↓97% PR	↓46% MR	↓61% PR
57	↓68% PR	↓77% PR	↓100% CR	↓100% CR	↓42% MR	↓56% PR
58	↓41% MR	↓46% MR	↓57% PR	↓73% PR	↓54% PR	↓68% PR

Pt ID	Bidimentional % reduction / response USS	Tridimention-al % reduction / response USS	Bidimentio-nal % reduction / response Calliper	Tridimenti-onal % reduction / response Calliper	Bidimentio-nal % reduction / response Mammo	Tridimen-tional % reduction / response Mammo
59	↓75% PR	↓86% PR	↓64% PR	↓78% PR	↓58% PR	↓73% PR
60	↓50% PR	↓45% MR	↓66% PR	↓80% PR	↓51% PR	↓63% PR
61	↓84% PR	↓91% PR	↓89% PR	↓96% PR	↓63% PR	↓77% PR
62	↓58% PR	↓75% PR	↓100% CR	↓100% CR	↓18% NC	↓25% MR
63	↓48% MR	↓59% PR	↓34% MR	↓47% MR	↓12% NC	↓17% NC
64						
65	↑ 4% NC	↓11% NC	↓29% MC	↓41% MR	↑ 7 % NC	↑ 10% NC
66	↓65% PR	↓50% PR	↓66% PR	↓81% PR	**Tumour not measurable**	**Tumour not mea**
67	↓56% PR	↓78% PR	↓56% PR	↓71% PR	↓56% PR	↓70% PR
68	↑4% NC	↑23% NC	↓100% CR	↓100% CR	↓64% PR	↓77% PR
69	↓80% PR	↓76% PR	↓80% PR	↓91% PR	↓66% PR	↓80% PR
70	Too large to visualise	Too large to visualise	↓51% PR	↓67% PR	↓56% PR	↓71% PR
71	↓84% PR	↓95% PR	↓89% PR	↓96% PR	↓57% PR	↓72% PR
72	Too large to visualise on USS	Too large to visualise on USS	↓65% PR	↓84% PR	Not done – fungating lesion	Not done fungating lesion
73	↓73% PR ↓33% MR	↓87% PR ↓48% MR	↓78% PR NA	↓89% PR NA	↓31% PR NA	↓49% PR NA
74	↓42% MR	↓57% PR	↓42% MR	↓57% PR	↓21% NC	↓30% MR
75	↓18% NC	↓26% MR	↓100% CR	↓100% CR	↓63% PR	↓77% PR
76	↓29% MR	↓33% MR	↓39% MR	↓50% PR	58% PR	↓71% PR
77	↓37% MR ↓80% PR	↓51% PR ↓90% PR	↓56% PR ↓64% PR	↓71% PR ↓78% PR	↓15% NC ↓61% PR	↓21% NC ↓76% PR
78	↓60% PR ↓71% PR	↓75% PR ↓86% PR	↓32% MR ↓100% CR	↓44% MR ↓100% CR	↓19% NC ↓46% MR	↓19% NC ↓20% NC
79	↓29% MR	↓42% MR	↓76% PR	↓90% PR	↓5% NC	↓7% NC
80	NA	NA	NA	NA	NA	NA
81	↓30% MR	↓11% NC	↑11% NC	↑15% NC	↓35% MR	↓48% MR
82	↑2% NC ↑33% PD	↑15% NC ↑2% NC	↑ 51% PD NA	↑85% PD NA	↑31% PD NA	↑49% PD NA
83	↓76% PR	↓84% PR	↓100% CR	↓100% CR	↓43% MR	↓57% PR
84	↓28% MR	↓16%PR	↓18% NC	↓25% PR	↓38% MR	↓51% PR
85	↓83% PR	↓93% PR	↓70% PR	↓84% PR	↓75% PR	↓86% PR
86	↑ 109% PD	↑ 209% PD	↑ 9% NC	↑ 14% NC	↑ 146% PD	↑ 278% PD
87	↓82% PR	↓92% PR	↓84% PR	↓93% PR	↓69% PR	↓83% PR
88	Not part of clinical measurements	----------	----------	----------	----------	-------
89	↓67% PR	↓85% PR	↓100% CR	↓100% CR	↓31% MR	↓41% MR

Pt ID	Bidimentional % reduction / response USS	Tridimentional % reduction / response USS	Bidimentio-nal % reduction / response Calliper	Tridimentio-nal % reduction / response Calliper	Bidimentional % reduction / response Mammo	Tridimen-tional % reduction / response Mammo
90	↓70% PR	↓80% PR	↓100% CR	↓100% CR	↓65% PR	↓79% PR
91	↓28% MR	↓22% NC	↓19% NC	↓27% MR	↓42% MR	↓55% PR
92	↑25% PD ↑71% PD	↑47% PD ↑103% PD	↑25% PD NA	↑42% PD NA	↑59% PD NA	↑101% PD NA
93	↓52% PR	↓52%PR	*↓49% MR	*↓65% PR	Not assessable on film	Not assessable
94	↓71% PR	↓74% PR	↓49% MR	↓64% PR	↑2.3% NC	↓3% NC
95	↓16% NC	↓4% NC	↓22% NC	↓31% NC	10% NC	15% NC
96	↓80% PR ↑41% PD	↓87% PR ↑24% NC	↓100% PR ↑29% PD	↓100% PR ↑46% PD	↓38% MR NA	↓52% PR NA
97	↓58% PR	↓67% PR	↓75% PR	↓88% PR	↓19% NC	↓19% NC
98	NA	NA	NA	NA	NA	NA
99	↓9% NC	↓23% NC	↓21% NC	↓30% MR	↓32% MR	↓46% MR
100	↓36% MR	↓35% MR	↓75% PR	↓88% PR	↓63% PR	↓79% PR
101	↓87% PR	↓92%PR	↓100% CR	↓100% CR	↓52% PR	↓63% PR
102	↓63% PR	↓80% PR	↓74% PR	↓86% PR	↓11% NC	↓16% NC
103	↓15% NC	↓8% NC	↓31% MR	↓44% MR	↓24% NC	↓40% MR
104	↑79% PD ↓6% NC	↑63% PD ↓16% NC	↑19% NC ↓38% MR	↑30% PD ↓51% PR	↓29% MR ↓11% NC	↓46% MR ↓20% NC
105	↓31% MR	↓37% MR	↓19% NC	↓27% MR	↑0.6% NC	↑0.6% NC
106	↓36% MR	↓47% MR	Cannot assess	Cannot assess	Cannot assess	Cannot assess
107	↓45% MR	↓56% PR	↓54% PR	↓69% PR	↓48% MR	↓63% PR
108	↓80% PR ↑18% NC	↓89% PR ↑19% NC	↓40% MR not assessable	↓53% PR not assessable	↓44% MR ↓10% NC	↓55% PR ↓14% NC
109	↓42% MR	↓32% MR	↓52% PR	↓67% PR	↓40% MR	↓55% PR
110	↓43% (MR	↓76% PR	↓100% CR	↓100% CR	↓63% MR	↓78% MR
111	↓62% PR	↓72% PR	↓100% CR	↓100% CR	↓54% PR	↓70% PR
112	↓93% PR	↓95% PR	↓100% CR	↓100% CR	↓69% PR	↓81% PR
113	NA	NA	NA	NA	NA	NA
114	↓61% PR	↓84% PR	Cannot assess	Cannot assess	Cannot assess	Cannot assess
115	↓87% PR	↓90% PR	↓100% CR	↓100% CR	↓ 74% PR	↓85% PR
116	↓85% PR	↓88% PR	↓100% CR	↓100% CR	↓43% MR	↓50% PR
117	↓60% PR	↓73% PR	↓43% MR	↓57% PR	↓67% PR	↓83% PR
118	↓41% MR	↓58% PR	↓51% PR	↓66% PR	↓56% PR	↓70% PR
119	↓76% PR	↓86% PR	↓61% PR	↓77% PR	↓45% MR	↓60% PR
120	↓67% PR	↓85% PR	↓55% PR	↓69% PR	↓64% PR	↓77% PR
121	↓22% NC	↓53% MR	*↓30% MR	*↓43% MR	↑18% NC	↑30% PD
122	↓36% MR	↓12% NC	↓3% NC	↓4% NC	↓49% MR	↓63% PR
123	↓20% NC	↓53% PR	↓63% PR	↓78% PR	↓58% PR	↓72% PR
124	↑5% NC	↓0.4% NC	*↓16% NC	*↓23% NC	↓12% NC	↓3% NC
125	↓81% PR	↓89% PR	↓82% PR	↓92% PR	↓66% PR	↓80% PR

Pt ID	Bidimentional % reduction / response USS	Tridimentional % reduction / response USS	Bidimentio-nal % reduction / response Calliper	Tridimention -al % reduction / response Calliper	Bidimentio-nal % reduction / response Mammo	Tridimen-tional % reduction / response Mammo
126	↓82% PR ↓69% PR	↓95% PR ↓93% PR	↓100% CR NA	↓100% CR NA	↓88% PR ↓67% PR	↓96% PR ↓81% PR
127	↓68% PR ↓15% NC ↓47% MR	↓81% PR ↓41% MR ↓48% MR	↓24% NC ↑70% PD ↓51% PR	↓35% MR ↑94% PD ↓65% PR	↓65% PR ↓62% PR unable to assess central	↓78% PR ↓77% PR unable to assess central
128	NA	NA	NA	NA	NA	NA
129	↓52% PR	↓72% PR	↓61% PR	↓77% PR	↓53% PR	↓67% PR
130	↓69% PR	↓70% PR	↓50% PR	↓65% PR	↓53% PR	↓ 67% PR
131	↑93% PD	↑28% PD	↓37% MR	↓49% MR	↑22% NC	↑36% PD
132	↓50% PR	↓61% PR	↓61% PR	↓76% PR	↓48% MR	↓63% PR
133	↓76% PR	↓87% PR	↓83% PR	↓93% PR	↓42% MR	↓55% PR
134	NA	NA	NA	NA	NA	NA
135	↓27% MR	↓45% MR	↓79% PR	↓91% PR	↓45% MR	↓59% PR
136	↓43% MR	↓42% MR	↓42% MR	↓56% PR	↓24% NC	↓ 34% MR
137	↓45% MR	↓46% MR	↓100% CR	↓100% CR	↓68% PR	↓82% PR

Appendix 15: Comparison of Initial (pre) and final (post) ultrasound (USS) tumour measurements compared with final measured tumour size from pathology specimen (in cm) where applicable

Case	Patient No	Pre USS	Post USS	PATH
1	2	7.29	2.66	3.61
2	3	13.36	2.24	4
3	6	14.52	7.56	7.84
4	7	4.8	0.78	6.25
5	8	5.6	3.24	9
6	10	5.28	0.55	2.56
7	12	7.83	5.52	CORES
8	15	13.3	1.14	CORES
9	17	10.24	3.22	4
10	18	3.36	1.82	CORES
11	20	18.48	7.2	6.25
12	23	12.25	6.65	6.25
13	24	23.32	5	6.25
14	25	6.75	1.09	9
15	26	4.1	2.1	2.56
16	27	7.75	5.75	3.61
17	34	5.76	0.56	CORES
18	35	2.08	1.24	2.25
19	38	8.7	5.67	4
20	43	4.8	3.6	4
21	44	6.09	0.9	8.7
22	48	10.5	4.18	6.76
23	50	11.2	5.44	5.76
24	51	7.8	1.08	6.76
25	52	9.24	4.59	10.24
26	53	7.68	2.38	1.44
27	56	5.98	3.23	3.24
28	57	3.23	1.04	11.56
29	58	7.02	4.18	6.25
30	59	9.57	2.4	7.84
31	61	4.4	0.72	5.76
32	62	2.6	1.1	4.41
33	63	5	2.63	2.89
34	65	5.78	6	7.29
35	66	9.1	3.23	CORES
36	67	3.15	1.39	2.56
37	69	4.14	0.84	1.44
38	70	TOO	LARGE	CORES
39	71	12.48	1.96	>36 (lob)
40	72	TOO	LARGE	CORES

41	74	2.89	1.69	6.25
42	75	0.57	0.47	0.3
43	76	10.73	7.6	CORES
44	77	8	5.06	4.41
45	78	1.82	0.52	2.56
46	79	2.88	2.04	5.29
47	84	4.75	3.42	5.29
48	85	4.14	0.69	0.36
49	86	7.02	14.7	16
50	87	6.5	1.17	0.81

Appendix 16: Clinical and pathological response categories for the 62 patients in the biological subgroup

Pt no	Case	Clinical Response	Pathological response
1	2	PR	PR
2	3	PR	PR
3	6	PR	PR
4	7	PR	PR
5	10	PR	PR
6	12	PR	PR
7	15	PR	PR
8	17	PR	PR
9	20	PR	PR
10	23	PR	PR
11	24	PR	PR
12	25	PR	PR
13	26	PR	PR
14	27	NC	NC
15	34	PR	PR
16	35	PR	PR
17	38	MR	PR
18	43	MR	MR
19	48	PR	PR
20	50	PR	PR
21	51	PR	PR
22	52	PR	NC/MR
23	53	PR	PR
24	56	PR	PR
25	57	PR	NC
26	58	MR	NC/MR
27	59	PR	PR
28	61	PR	NC/MR
29	63	PR	PR
30	66	PR	PR
31	67	PR	PR
32	69	PR	PR
33	74	PR/MR	PR/MR
34	76	MR	NA
35	77	PR	PR
36	78	PR	PR
37	79	MR	PR
38	84	MR	NC
39	89	PR	PR
40	90	PR	PR
41	93	PR	NC/MR
42	94	PR	PR

43	95	NC	NC
44	97	PR	PR
45	100	MR	MR
46	102	PR	PR
47	105	MR	PR
48	107	PR	PR
49	109	MR	NC
50	110	PR	PR
51	111	PR	PR
52	115	PR	PR
53	116	PR	PR
54	117	PR	PR
55	118	PR	NC
56	119	PR	PR
57	120	PR	PR
58	121	MR	MR
59	125	PR	NC/MR
60	130	PR	PR
61	132	PR	PR
62	136	MR	PR

Appendix 17: Raw data for ER for biological subgroup

Pt no	Audit no	Pre		10-14d		3 m	
		ER p	ER i	ER p	ER i	ER p	ER i
1	2	5	2	5	3	5	2
2	3	5	3	5	3	5	2
3	6	5	3	5	3	5	3
4	7	5	3	5	3	5	2
5	10	5	3	5	3	5	2
6	12	5	2	5	3	5	3
7	15	5	3	5	3	5	3
8	17	5	2	5	3	5	2
9	20	5	3	5	2	5	2
10	23	5	3	5	3	5	3
11	24	5	3	5	3	5	2
12	25	5	3	5	3	5	3
13	26	5	3	5	2	5	2
14	27	5	3	5	3	5	2
15	34	5	3	5	3	5	3
16	35	5	3	5	2	5	2
17	38	5	3	5	3	5	3
18	43	5	3	5	3	5	2
19	48	5	3	5	3	5	3
20	50	5	3	5	3	5	2
21	51	5	3	5	3	5	3
22	52	5	3	5	2	5	2
23	53	5	3	5	3	5	2
24	56	5	3	5	3	5	3
25	57	5	2	5	2	5	2
26	58	5	3	5	3	5	3
27	59	5	3	5	2	5	3
28	61	5	3	5	3	5	3
29	63	5	3	5	3	5	2
30	66	5	3	5	2	5	3
31	67	5	2	5	2	5	2
32	69	5	2	5	2	5	2
33	74	5	3	5	3	5	3
34	76	5	3	5	3	5	2
35	77	5	2	5	2	5	2
36	78	5	2	5	3	5	3
37	79	5	2	5	3	5	3
38	84	5	3	5	3	5	2
39	89	5	3	5	3	5	2
40	90	5	3	5	3	5	2
41	93	5	2	5	2	5	2

Pt	Audit						
no	no	Pre		10-14d		3 m	
		ER p	ER i	ER p	ER i	ER p	ER i
42	94	5	3	4	2	4	2
43	95	5	3	5	3	5	2
44	97	5	2	5	2	5	3
45	82	5	3	5	3	5	2
46	77	5	3	5	3	5	2
47	105	5	3	5	3	5	2
48	107	5	3	5	3	5	3
49	109	5	2	5	3	5	3
50	110	5	2	5	2	5	2
51	111	5	3	5	3	5	3
52	115	5	2	5	3	5	2
53	116	5	3	5	3	5	3
54	117	5	3	5	3	5	3
55	118	5	3	5	3	5	3
56	119	5	2	5	3	4	1
57	120	5	2	5	3	5	3
58	121	5	3	4	2	3	2
59	125	5	3	5	3	5	3
60	130	5	3	5	3	5	3
61	132	5	3	5	3	5	3
62	136	5	3	5	3	5	2

Appendix 18: Raw data for PgR for biological subgroup

Pt no	Audit no	Pre		10-14d		3 m	
		PgR p	PgR i	PgR p	PgR i	PgR p	PgR i
1	2	2	2	0	0	0	0
2	3	0	0	0	0	0	0
3	6	5	3	4	1	4	1
4	7	3	2	0	0	0	0
5	10	5	3	5	3	5	2
6	12	5	3	5	3	5	2
7	15	3	1	0	0	0	0
8	17	5	2	5	2	5	2
9	20	0	0	0	0	1	1
10	23	4	1	0	0	1	1
11	24	5	3	5	3	5	2
12	25	5	3	0	0	0	0
13	26	5	2	5	3	5	2
14	27	5	3	5	2	5	1
15	34	5	3	5	2	4	1
16	35	5	3	3	1	3	2
17	38	5	3	5	2	5	3
18	43	3	2	1	1	1	1
19	48	5	3	5	3	5	2
20	50	3	1	1	1	0	0
21	51	4	1	0	0	0	0
22	52	3	1	0	0	0	0
23	53	4	2	3	1	0	0
24	56	5	2	0	0	0	0
25	57	0	0	0	0	0	0
26	58	4	2	0	0	0	0
27	59	4	2	2	1	0	0
28	61	4	2	0	0	0	0
29	64	5	2	0	0	0	0
30	66	4	2	0	0	0	0
31	67	3	2	0	0	0	0
32	69	5	3	2	2	3	2
33	74	5	2	4	2	0	0
34	76	4	2	0	0	0	0
35	77	5	2	5	2	3	1
36	78	5	3	0	0	0	0
37	79	0	0	0	0	0	0
38	84	4	2	0	0	0	0
39	89	5	3	1	1	0	0
40	90	1	2	1	1	0	0
41	93	0	0	0	0	0	0

Pt	Audit						
no	no	Pre		10-14d		3 m	
		PgR p	PgR i	PgR p	PgR i	PgR p	PgR i
42	94	0	0	0	0	0	0
43	95	3	1	0	0	0	0
44	97	5	2	0	0	0	0
45	100	2	2	0	0	0	0
46	102	2	2	0	0	0	0
47	105	0	0	0	0	0	0
48	107	5	3	4	3	5	3
49	109	2	2	0	0	0	0
50	110	4	2	2	2	2	1
51	111	5	3	1	2	0	0
52	115	5	2	3	1	0	0
53	116	4	2	0	0	0	0
54	117	3	3	0	0	0	0
55	118	3	3	0	0	0	0
56	119	3	2	3	2	3	2
57	120	5	2	0	0	0	0
58	121	3	2	0	0	0	0
59	125			0	0	0	0
60	130	5	3	4	2	0	0
61	132	5	3	3	1	0	0
62	136	5	3	5	2	4	2

Appendix 19: Raw data for proliferation from Letrozole Audit

Case	Pre	10-14 d	3month	%decrease at 10-14d	%increase at 10-14d	%decrease at 3months	%increase at 3 months	%decrease 10-14 to 3m	%increase 10-14 to 3m
2	19.16	11.12	7.07	41.97%		63.20%		21.23%	
3	28.14	0.7	0.28	97.51		99		1.49	
6	7.51	0.45	0	94.01		100		5.99	
7	12.16	1.84	0.64	84.87		94.79		10.1	
10	9.83	2.4	0.08	75.59		99.18		23.59	
12	8.15	1.64	5.25	79.88		35.9			43.98
15	21.8	1.3	0	94.04		100		5.96	
17	6.34	3.91	0.25	38.33		96.06		57.73	
20	17.79	32.87	22.88		83.63		28.61	55.02	
23	4.98	4.55	1.18	8.64		76.31		67.67	
24	10.8	1.75	2.29	83.8		78.79			14.08
25	14.48	7.26	5.32	49.97		63.26		13.29	
26	19.73	1.47	0.93	92.55		95.29		2.74	
27	22.06	7.74	3.21	66.69		85.45		18.76	
34	17.57	8.93	0.8	49.18		95.45		46.27	
35	11.97	2.95	1.08	75.54		90.98		15.44	
38	15.78	4.33	8.19	72.57		48.1			24.47
43	10.08	3.59	1.98	74.49		81.36		6.87	
48	11.69	1.65	0	85.89		100		14.11	
50	19.76	3.49	15.79	82.34		10.22			72.12
51	15.53	13.4	16.8	13.72			8.17		14.8
52	17.85	10.69	9	40.12		49.58		9.46	
53	6.27	2.84	0.68	54.71		89.16		49.04	
56	1.64	1.09	0	33.6		100		66.4	
57	22.32	24.78	15.54		11.02	30.38		31.49	
58	24.06	2.67	0.84	88.91		96.51		7.6	
59	5.25	2.36	0.22	55.05		95.51		40.46	
61	9.43	0	0.77	100		99.23			0.77
63	15.12	1.96	3.35	87.04		77.85			9.19
66	5.71	3.25	1.09	43.09		80.91		55.83	
67	15.66	0.49	1.1	96.88		92.98			4.1

Case	Pre	10-14d	3month	%decrease at 10-14d	%increase at 10-14d	%decrease at 3months	%increase at 3 months	%decrease 10-14 to 3m	%increase 10-14 to 3m
69	9.37	2.73	0.36	70.87		96.16		28.75	
74	9.3	18.85	5.02		102.6	44.1			
76	10.28	1.03	0.64	89.99		93.78		3.79	
77-L	9.27	2.66	3.39	71.33		63.44			7.85
78-L	11.84	2.9	1.94	75.51		83.62		8.11	
79	17.12	0.66	0	96.14		100		3.86	
84	31.82	21.86	18.22	31.31		42.74		11.43	
87	16.14	19.71			22.11				
89	6.51	0.74	0	88.64		100		11.36	
90	22.35	9.81	3.11	56.11		86.09		29.98	
93	4.76	1.91	1.27	59.88		73.32		13.44	
94	28.86	16.11	22.93	44.18		20.55			23.63
95	8.63	1.71	12.35	80.19			43.1		
97	5.86	1.07	0	81.75		100		18.25	
100	4.03	0	0	100		100		0	
102	3.51	0.53	0	84.9		100		15.1	
105	10.98	0.93	2.24	91.54		79.6			11.94
107	8.81	0	0.54	100		93.87			6.13
109	27.79	20.01	25.46	28		8.39			19.61
110	17.44	1.86	0	89.34		100		10.66	
111	8.76	1.26	0.65	85.62		92.58		6.96	
115	28.18	1.57	1.47	94.42		94.78		0.36	
116	9.45	2.36	0.43	75.03		95.45		20.42	
117	23.18	1.33	2.73	94.27		88.23			6.04
118	21.28	3.22	12.24	84.67		42.49			42.18
119	5.06	0.41	0	91.9		100		8.1	
120	28.26	12.83	20.02	54.61		29.16			25.45
121	15.38	14.33	2.29	6.83		85.11		78.28	
125	10.74	8.93	13.47	16.86			20.87		42.27
130	23.64	2.76	1.18	88.33		95		6.67	
132	28.24	2.88	1.08	89.81		96.18		6.37	
136	13.85	1.38	2.11	90.04		84.76			5.28

Appendix 20: Raw data for Ki67 for biological subgroup

Patient number	Audit number	Ki-67 (%)		
		Pre	10-14d	3 mth
1	2	19.16	11.12	7.07
2	3	28.14	0.7	0.28
3	6	7.51	0.45	0
4	7	12.16	1.84	0.64
5	10	9.83	2.4	0.08
6	12	8.15	1.64	5.25
7	15	21.8	1.3	0
8	17	6.34	3.91	0.25
9	20	17.79	32.87	22.88
10	23	4.98	4.55	1.18
11	24	10.8	1.75	2.29
12	25	14.48	7.26	5.32
13	26	19.73	1.47	0.93
14	27	22.06	7.74	3.21
15	34	17.57	8.93	0.8
16	35	11.97	2.95	1.08
17	38	15.78	4.33	8.19
18	43	10.08	3.59	1.98
19	48	11.69	1.65	0
20	50	19.76	3.49	15.79
21	51	15.53	13.4	16.8
22	52	17.85	10.69	9
23	53	6.27	2.84	0.68
24	56	1.64	1.09	0
25	57	22.32	24.78	15.54
26	58	24.06	2.67	0.84
27	59	5.25	2.36	0.22
28	61	9.43	0	0.77
29	64	15.12	1.96	3.35
30	66	5.71	3.25	1.09
31	67	15.66	0.49	1.1
32	69	9.37	2.73	0.36
33	74	9.3	18.85	5.02
34	76	10.28	1.03	0.64
35	77	9.27	2.66	3.39
36	78	11.84	2.9	1.94
37	79	17.12	0.66	0
38	84	31.82	21.86	18.22
40	89	6.51	0.74	0
41	90	22.35	9.81	3.11
42	93	4.76	1.91	1.27
43	94	28.86	16.11	22.93

Section 9: Publications

Peer reviewed journals

Estrogen independent proliferation is present in the majority of estrogen receptor positive (ER+) HER2 gene amplified primary breast cancers after letrozole exposure despite frequent tumor regression during neoadjuvant treatment Ellis MJ, Tao Y, Young O, White S, Proia AD, **Murray J**, Renshaw L, Faratian D, Thomas J, Dowsett M, Krause A, Evans DB, Miller WR, Dixon JM. *J Clin Oncol* ; June 2006

Neoadjuvant endocrine therapy models. **Murray J**, Miller WR, Dixon JM. *Methods Mol Med* 2006;120 :489-502

Proliferation, steroid receptors and clinical/ pathological response in breast cancer treated with letrozole. Miller WR, White S, Dixon JM, **Murray J**, Renshaw L, Anderson TJ. *British Journal of Cancer* (2006) 94(7), 1051 -1056

Aromatase Inhibitors: Cellular and molecular effects. Miller WR, Anderson TJ, White S, Larionov A, **Murray J**, Evans D, Krause, Dixon JM. *J. Steroid Biochem Molecular Biol 95 (*2005)83-89

Surgical issues surrounding use of aromatase inhibitors. Dixon JM, Renshaw L, **Murray J**, Macaskill EJ, Young O, Miller WR. *Journal of Steroid Biochemistry and Molecular Biology* 2005; 95: 97-103.

Neoadjuvant tamoxifen and aromatase inhibitors: Comparisons and clinical outcomes. Dixon JM, **Jackson J**, Miller WR. *J. Steroid Biochem Molecular Biol.* 2003 Sep;86(3-5) : 295-9

The therapeutic potential of aromatase inhibitors. Miller WR, **Jackson J**. *Expert Opin on Investig Drugs* 2003; 12(3): 337-351

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JOURNAL OF CLINICAL ONCOLOGY ORIGINAL REPORT

Estrogen-Independent Proliferation Is Present in Estrogen-Receptor *HER2*-Positive Primary Breast Cancer After Neoadjuvant Letrozole

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Authors' disclosures of potential conflicts of interest and author contributions are found at the end of this article.

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0732-183X/06/2419-3019/$20.00

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ABSTRACT

Purpose
To investigate the impact of human epidermal growth factor receptor (*HER*) 1 and *HER2* gene amplification on endocrine therapy responsiveness, a fluorescence in situ hybridization (FISH) study was conducted on tumor samples from 305 postmenopausal patients with stage II and III estrogen receptor (ER) –positive (ER ≥ 10%) breast cancers treated on two independent neoadjuvant endocrine therapy trials.

Patients and Methods
FISH analysis focused on *HER1* and/or *HER2* immunohistochemistry (IHC) -positive patients and a random selection of *HER1/2* IHC-negative patients. *HER2* FISH status was correlated with response and changes in the proliferation marker Ki67.

Results
HER1 was rarely amplified (< 1%), and *HER2* amplification was observed in 9.2% of patients. Letrozole response by clinical measurement (71% *HER2* FISH positive *v* 71% *HER2* FISH negative), mammogram (44% *HER2* FISH positive *v* 47% *HER2* FISH negative), or ultrasound (47% *HER2* FISH positive *v* 54% *HER2* FISH negative) was not impaired by *HER2* FISH-positive status. In contrast, *HER2* FISH-positive tumors showed higher histologic grade ($P = .009$), higher pretreatment Ki67 ($P = .005$), and less Ki67 suppression after letrozole when compared with *HER2* FISH-negative tumors ($P = .0001$). Similar observations regarding Ki67 were made in a smaller cohort of tamoxifen-treated tumors.

Conclusion
Neoadjuvant letrozole is clinically effective in ER-positive *HER2* FISH-positive tumors, indicating sensitivity to short-term estrogen deprivation. However, continued proliferation despite ongoing letrozole or tamoxifen treatment in the majority of ER-positive *HER2* FISH-positive samples (88%) could imply therapeutic resistance that may manifest later in the clinical course of the disease. Discordance between clinical and biomarker findings in this study serves to emphasize the need for surrogate end point validation in neoadjuvant endocrine trials through correlation with information on long-term outcomes.

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INTRODUCTION

Most studies[1] have concluded that estrogen receptor (ER) –positive breast cancers exhibiting human epidermal growth factor receptor (HER) 2 protein overexpression have diminished responsiveness to tamoxifen. The exceptions have tended to occur in the setting of adjuvant studies where chemotherapy use may obscure the interaction.[2] In contrast, a neoadjuvant study that compared tamoxifen with letrozole indicated that preoperative regression of ER-positive tumors in response to 4 months of letrozole was not impeded by overexpression of *HER1* and/or *HER2*.[3] A similar conclusion was reached in a preoperative study of anastrozole versus tamoxifen,[4] but the size of the ER-positive, *HER2*-positive subsets in both these studies was too small for definitive conclusions. Small sample size is a consistent problem with the literature because ER-positive, *HER2*-positive tumors are uncommon, with only 10% of ER-positive disease showing evidence of *HER2* gene amplification.[5] Nonetheless, studies that focus on the correct endocrine approach for ER-positive *HER2*-positive disease are important

because half of *HER2* fluorescence in situ hybridization (FISH) –positive primary breast cancers exhibit some degree of hormone receptor expression.

Since our initial publication,[3] FISH testing has replaced immunohistochemistry (IHC) as the gold standard for *HER2* assessment. Therefore, we re-examined our tumor bank using *HER1* and *HER2* FISH probes. Treatment-associated changes in Ki67 staining are also presented because this proliferation biomarker provides useful additional information on endocrine responsiveness in the preoperative setting.[6,7] To increase our sample size, we combined clinical information and tumor samples from two independent studies of preoperative endocrine therapy.

PATIENTS AND METHODS

Study Population and Tumor Bank

The P024 protocol was a double-blind randomized trial that compared 4 months of preoperative letrozole with tamoxifen in postmenopausal women with stage II and III breast cancers who were ineligible for breast conservation. The clinical findings and tumor bank characteristics have been described previously.[3,6,8] The P024 letrozole cohort was expanded with an additional 106 samples from a consecutive series of patients treated with preoperative letrozole in the Edinburgh Breast Unit.[9] Eligibility criteria and tumor assessments for patients treated on the Edinburgh study were similar to those used in the letrozole P024 trial. However, some of the tumors treated in the Edinburgh cohort were smaller than 3 cm, and the Edinburgh patients were treated for a shorter period (typically approximately 12 weeks rather than 16 weeks). All studies were conducted with approval from the institutional review boards or ethics committees of the institutions involved in either enrolling patients or in sample analysis.

Biomarker Analysis: IHC

Details of the *HER1*, *HER2*, ER, progesterone receptor (PgR), and Ki67 IHC methodologies used for the P024 samples have been published previously.[3,6] When the additional samples were received from Edinburgh, they were initially assessed by *HER2* IHC using the 3B5 antibody using the same protocol reported for P024.[3] Data on ER, PgR, Ki67, and histologic grade were already available from the Edinburgh database. ER and PgR IHC used clinical grade reagents and controls, and Ki67 IHC was based on the MIB1 antibody (diluted × 50; Europath Ltd, Cornwall, United Kingdom). Reactivity was detected according to the method described by Going.[10]

Biomarker Analysis: FISH

HER1 and *HER2* FISH analyses were conducted using PathVysion *HER1* and *HER2* DNA probe kits according to the manufacturer's instructions (Vysis, Downers Grove, IL). *HER2* FISH scoring with a fluorescent microscope (Olympus BX51; Olympus, Tokyo, Japan), was conducted manually by a board-certified pathologist (A.D.P.) who was blind to treatment, biomarker status, and clinical outcome. One hundred forty-three patients were subjected to *HER2* FISH analysis, including all patients who exhibited *HER2* 2+ and 3+ IHC staining in either the pre- or post-treatment sample. No samples (zero of 70 samples) of *HER2* gene amplification were observed from a random sample of *HER2* IHC-negative (0 or 1+) samples (from the P024 study). Twenty-seven percent of samples exhibiting 2+ staining were found to be FISH positive (16 of 60 samples), and of samples with 3+ staining, 86% (12 of 14 samples) were positive. FISH analysis was not conducted on the remaining IHC-negative samples because the 3B5 antibody efficiently screened out tumors with a low chance of *HER2* gene amplification.

Biostatistical Analysis

All the *P* values reported are two sided, and a $P = .05$ was considered significant. No adjustment for multiple testing was conducted. Fisher's exact and χ^2 tests were used to define associations between *HER2* gene amplification status and clinical, mammography, ultrasound, and cell cycle responses. The nonparametric Mann-Whitney *U* test was applied to compare differences in Ki67 changes between *HER2*-positive and *HER2*-negative tumors. The differences between baseline and post-treatment Ki67 values within each *HER2* group (amplified or not amplified) were assessed by the Wilcoxon signed rank test. The 95% CI of the geometric Ki67 mean was calculated to show the size of effects in pair-wise comparisons. All statistical analyses were performed using SAS 9.1.2 (SAS Institute Inc, Cary, NC).

Table 1. Baseline Clinical and Biomarker Characteristics of the *HER2* FISH-Negative Population and the *HER2* FISH-Positive Population

	HER2 FISH Positive		*HER2* FISH Negative		
Baseline Characteristic	Median	Range	Median	Range	*P*
Age, years	71	51-88	71	44-93	.3710
Clinical size, cm	4.6	1.5-8.0	4.2	2-15	.7761
Ultrasound size, cm	3	1.6-8.0	2.9	0.3-14.0	.3216
Mammogram size, cm	4	1.4-8.0	3.5	1.2-14.0	.2845
ER, Allred	8	4-8	8	4-8	.2811
PgR, Allred	4	0-8	5	0-8	.1205
Ki67, %	16.6	0-53.6	7.7	0-53.8	.005
Histologic grade, %					
1	0		14.6		
2	53.8		58.6		
3	46.2		26.8		.0088

Abbreviations: *HER2*, human epidermal growth factor receptor 2; FISH, fluorescence in situ hybridization; ER, estrogen receptor; PgR, progesterone receptor.

Definition of Cell Cycle Complete Response

Theoretically, if the tumor cell cycle is fully estrogen dependent, then tumor proliferation should be completely arrested by potent endocrine treatment. In published exploratory analyses, the antiproliferative responses to letrozole or tamoxifen were categorized as exhibiting a cell cycle complete response (CR) when the post-treatment Ki67 staining was 1% or less in the infiltrating component of the tumor.[11] We prospectively applied this cut point to the current data set.

RESULTS

Clinical and Biomarker Characteristics of ER-Positive, HER2 Gene–Amplified Tumors and HER1 FISH Analysis

HER2 gene amplification was detected in 9.2% of the samples (28 of 305 samples). The baseline clinical and biomarker characteristics of

Table 2. Baseline Clinical and Biomarker Characteristics According to Treatment With Letrozole or Tamoxifen

	Letrozole		Tamoxifen		
Baseline Characteristic	Median	Range	Median	Range	*P*
Age, years	73	44-93	67	48-89	.0017
Clinical size, cm	4.1	1.5-12.0	4.5	2.0-15	.3302
Ultrasound size, cm	2.8	0.3-8.0	3.0	1.3-14	.1547
Mammogram size, cm	3.4	1.2-8.35	3.6	1.4-14	.0938
ER, Allred	8	4-8	8	4-8	.1415
PgR, Allred	6	0-8	4	0-8	.0663
Ki67, %	8.7	0-53.8	7.8	0-42.9	.2459
Histologic grade					
1	14.4		10.8		
2	59.8		54.8		
3	25.8		34.4		.1207

Abbreviations: ER, estrogen receptor; PgR, progesterone receptor.

Table 3. Analysis of Clinical, Ultrasound, and Mammogram Response Data According to *HER2* FISH Status in Tamoxifen-Treated Patients

Response Category	*HER2* FISH Positive			*HER2* FISH Negative			
	No. of Responses	Total No.	%	No. of Responses	Total No.	%	Fisher *P*
Clinical	3	9	33	44	90	49	.49
Ultrasound	3	9	33	26	74	35	.99
Mammography	1	9	11	22	90	24	.68

Abbreviations: *HER2*, human epidermal growth factor receptor 2; FISH, fluorescence in situ hybridization.

the *HER2* FISH-positive versus *HER2* FISH-negative population are outlined in Table 1. The clinical characteristics of patients with ER-positive, *HER2* FISH-positive disease were not statistically different from patients with ER-positive, *HER2*-negative disease. In terms of the biomarker status, *HER2* FISH-positive tumors exhibited higher pretreatment Ki67 levels than *HER2* FISH-negative tumors (Mann-Whitney *U* test, $P = < .005$) and higher histologic grade ($P = .0088$). No statistically significant differences in clinical or biomarker status could be detected between the patients treated with tamoxifen and patients treated with letrozole, except that the tamoxifen-treated patients were slightly younger (Table 2). *HER1* FISH analysis was conducted on all patients from the P024 study (nine cases in total) who had HER1 based on IHC 2 or 3+ staining. Only one case with 3+ staining exhibited *HER1* gene amplification. Interestingly, this case exhibited gross amplification, with *HER1* gene copies too numerous to count.

Effect of HER2 *Gene Amplification on the Clinical Efficacy of Neoadjuvant Endocrine Therapy*

HER2 FISH analysis of specimens from the tamoxifen arm of the P024 study identified nine *HER2* FISH-positive tumors, and 90 were designated *HER2* FISH negative. Tamoxifen-treated tumors harboring *HER2* gene amplification exhibited lower response rates than *HER2* FISH-negative tumors by two of the three criteria examined (Table 3). These differences were not statistically significant, and the sample size provided limited statistical power to address the interaction. To more adequately examine the impact of *HER2* FISH status on the effectiveness of letrozole, additional samples from a single-arm neoadjuvant letrozole study were analyzed. The combined 202-sample letrozole data set included 17 *HER2* FISH-positive tumors and 185 *HER2* FISH-negative tumors. The letrozole response data, outlined in Table 4, indicated that the presence of *HER2* gene amplification did not substantially reduce the clinical effectiveness of neoadjuvant letrozole treatment, although the relatively small number of *HER2* FISH-positive tumors does not rule out a limited effect. The 95% CI for the letrozole clinical response in *HER2* FISH-positive tumors was 71% ± 23%. Thus, despite the sample size, a clinical response rate to letrozole of less than 48% in ER-positive, *HER2* FISH-positive disease can be excluded by these data. An exploratory analysis was conducted to compare the efficacy of tamoxifen and letrozole within subsets defined by *HER2* FISH status (Tables 5 and 6). These results should be interpreted with caution because the additional letrozole samples were not drawn from the original P024 double-blind randomized trial. Nonetheless, these findings do not contradict our earlier conclusions that letrozole is clinically more effective neoadjuvant therapy than tamoxifen. This advantage seems to be preserved in the *HER2* FISH-positive subset.[3,8]

HER2 *Gene Amplification and the Antiproliferative Effects of Letrozole*

An examination of the raw Ki67 data revealed that most *HER2* FISH-negative tumors showed a dramatic decline in Ki67 on treatment (Fig 1A). The geometric mean baseline Ki67 level in the *HER2* FISH-negative samples was 6.25 (95% CI, 5.16% to 7.58%); this level decreased to 0.68% after treatment (95% CI, 0.53% to 0.87%). This decrease was significant (Wilcoxon signed rank test, $P = .0001$; Fig 1C). In contrast, an examination of the *HER2* FISH-positive group showed that the impact of letrozole was blunted, with few samples decreasing close to 0% (Fig 1B). At baseline, the geometric mean Ki67 score in the *HER2* FISH-positive group was 14.73% (95% CI, 9.67% to 22.44%), and after treatment, the geometric mean decreased to 8.1% (95% CI, 4.16% to 15.75%). In contrast to the *HER2* FISH-negative samples, this decrease was not statistically significant (Fig 1D). The Mann-Whitney *U* test, which was applied to determine whether the paired Ki67 values in the *HER2* FISH-positive group were different from the values in the *HER2* FISH-negative cohort, was significant ($P = .0001$).

Table 4. Analysis of Clinical, Ultrasound, and Mammogram Response Data According to *HER2* FISH Status in Letrozole-Treated Patients

Response Category	*HER2* FISH Positive			*HER2* FISH Negative			
	No. of Reponses	Total No.	%	No. of Responses	Total No.	%	Mantel-Haenzel *P*
Clinical	12	17	71	131	185	71	.98
Ultrasound	8	17	47	91	170	54	.61
Mammography	7	16	44	84	178	47	.79

Abbreviations: *HER2*, human epidermal growth factor receptor 2; FISH, fluorescence in situ hybridization.

Table 5. Comparison of Clinical, Ultrasound, and Mammogram Response Data According to Treatment With Letrozole or Tamoxifen in *HER2* FISH-Negative Tumors

Response Category	Letrozole			Tamoxifen			Mantel-Haenzel *P*
	No. of Responses	Total No.	%	No. of Responses	Total No.	%	
Clinical	131	185	71	44	90	49	.0004
Ultrasound	91	170	54	26	74	35	.0083
Mammography	84	178	47	22	90	24	.0003

Abbreviations: *HER2*, human epidermal growth factor receptor 2; FISH, fluorescence in situ hybridization.

HER2 *Gene Amplification and the Antiproliferative Effects of Tamoxifen*

The 87 tamoxifen-treated *HER2* FISH-negative tumors with paired Ki67 data showed marked and statistically significant decreases with treatment (Fig 2A). The baseline geometric mean Ki67 level was 5.79% (95% CI, 4.43% to 7.56%); this level decreased to 1.29% (95% CI, 0.88% to 1.88%) on treatment (Wilcoxon signed rank test, $P = .0001$; Fig 2C). In contrast, the Ki67 score in the tamoxifen-treated *HER2* FISH-positive group did not show a statistically significant decrease; the baseline geometric mean was 6.9% (95% CI, 1.81% to 26.4%), and it was 6.5% (95% CI, 1.7% to 24.78%) in the post-treatment samples ($P = .65$; Fig 2B). A statistical comparison between the Ki67 changes in the *HER2* FISH-positive samples and the *HER2* FISH-negative samples did not reach significance ($P = .0925$; Fig 2D) presumably because of the small sample size; however, overall, the pattern of the results was similar to the letrozole result, which is consistent with prior conclusions that *HER2* gene amplification reduces the antiproliferative effects of tamoxifen as well as letrozole.[12]

HER2 *Gene Amplification Prevents Cell Cycle CR*

Table 7 examines the relationship between cell cycle CR and *HER2* gene amplification in tumors treated with letrozole. *HER2* FISH-positive tumors only occasionally exhibited a cell cycle CR (12%), underscoring the failure of letrozole to induce complete cell cycle arrest in most of the ER-positive *HER2*-positive tumors treated. In contrast, the majority (60%) of the *HER2* FISH-negative letrozole-treated tumors met the definition of a cell cycle CR at the time of surgery. This difference was statistically significant ($P = .0001$). A second exploratory analysis was conducted that restricted the comparison to samples in which the baseline Ki67 was at least 5% in both *HER2* FISH-negative tumors and *HER2* FISH-positive tumors in an attempt to adjust for the fact that *HER2* FISH-positive tumors had higher levels of Ki67 at baseline than *HER2* FISH-negative samples (Table 1). The relationship between cell cycle CR and *HER2* status remained significant in this subset ($P = .0012$; data not shown), indicating that the low rate of cell cycle CR in *HER2* gene–amplified tumors was not simply explained by the higher baseline Ki67 level. The relationship between cell cycle CR and *HER2* status on the tamoxifen arm was similar, with only one (11%) of nine *HER2* FISH-positive tumors exhibiting a cell cycle CR compared with 37 (42%) of 87 *HER2* FISH-negative tumors. In Table 8, a combined analysis is presented, drawing on the entire data set, regardless of whether the tumor was treated with letrozole or tamoxifen. The high level of correlation between the lack of cell cycle CR and the presence of a positive *HER2* FISH test ($P = .0001$) is consistent with the conclusion that the presence of *HER2* gene amplification generates resistance at the level of cell cycle progression regardless of which endocrine therapy is used.

DISCUSSION

This study was designed to investigate the relationship between *HER2* gene amplification and the efficacy of neoadjuvant endocrine therapy. By combining two sample sets, we confirmed that the clinical efficacy of letrozole in the neoadjuvant setting was not obviously compromised by the presence *HER2* gene amplification. This finding might be most helpful when considering a preoperative protocol for a postmenopausal patient with ER-positive *HER2* FISH-positive disease for whom a more toxic preoperative regimen is not suitable because of extreme age, frailty, or the presence of cardiac disease. The combination of letrozole and trastuzumab for the neoadjuvant treatment of postmenopausal women with ER-positive *HER2* FISH-positive disease is an appealing research question, although the relative scarcity of these tumors will make study accrual a challenge.

The finding that ER-positive *HER2* FISH-positive tumors and ER-positive *HER2* FISH-negative tumors respond similarly to letrozole at the clinical level is a striking contrast to the consistent finding that ER-positive *HER2* FISH-positive tumors are less sensitive to the cell cycle–inhibitory effects of letrozole (as well as other endocrine

Table 6. Comparison of Clinical, Ultrasound, and Mammogram Response Data According to Treatment With Letrozole or Tamoxifen in *HER2* FISH-Positive Tumors

Response Category	Letrozole			Tamoxifen			Fisher *P*
	No. of Responses	Total No.	%	No. of Responses	Total No.	%	
Clinical	12	17	71	3	9	33	.10
Ultrasound	8	17	47	3	9	33	.68
Mammography	7	16	44	1	9	11	.18

Abbreviations: *HER2*, human epidermal growth factor receptor 2; FISH, fluorescence in situ hybridization.

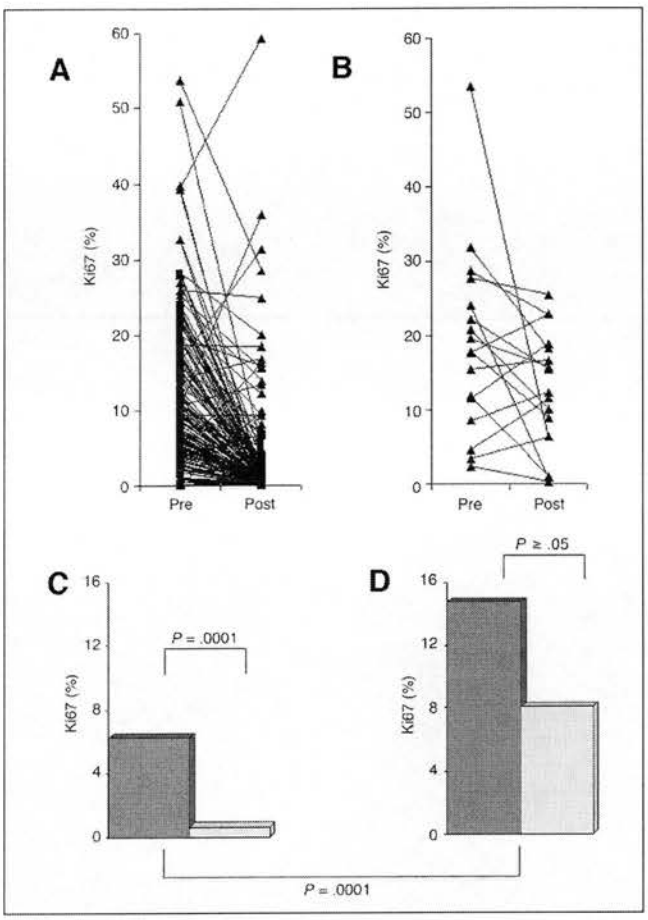

Fig 1. Paired Ki67 data before and after letrozole therapy according to human epidermal growth factor receptor 2 (*HER2*) fluorescence in situ hybridization (FISH) status: (A) Ki67 values in the *HER2* FISH-negative group; (B) Ki67 values in the *HER2* FISH-positive group; geometric mean levels in the (C) *HER2* FISH-negative group and (D) *HER2* FISH-positive group.

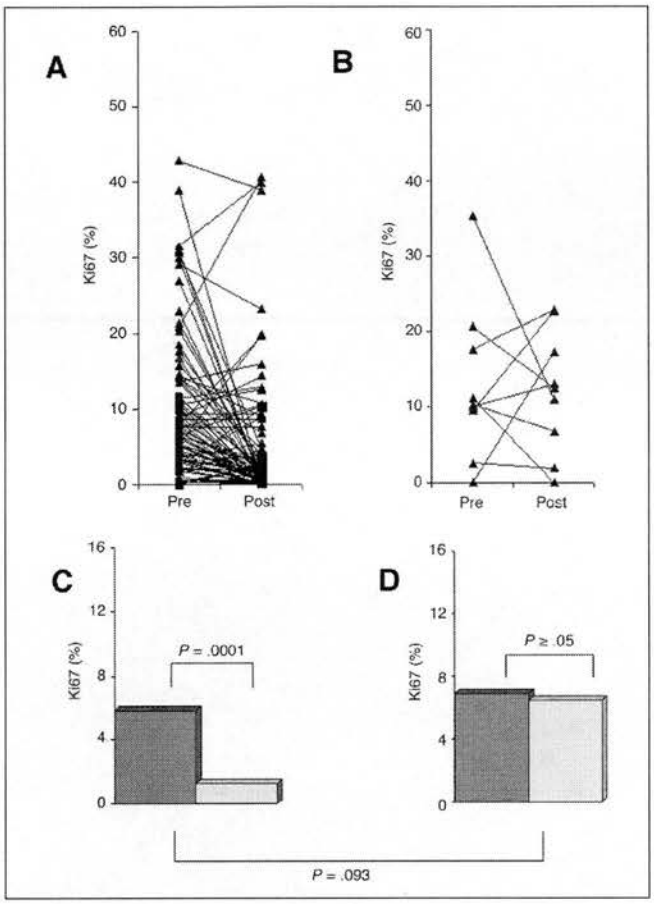

Fig 2. Paired Ki67 data before and after tamoxifen therapy according to human epidermal growth factor receptor 2 (*HER2*) fluorescence in situ hybridization (FISH) status: (A) Ki67 values in the *HER2* FISH-negative group; (B) Ki67 values in the *HER2* FISH-positive group; geometric mean levels in the (C) *HER2* FISH-negative group and (D) *HER2* FISH-positive group.

therapies). Dowsett et al[12] have reported similar Ki67 results after a much briefer exposure to a selective estrogen receptor modulator or an aromatase inhibitor (several weeks only). Together, these results suggest that the disparity between the data on tumor regression and Ki67 changes in the present study may be a primary influence of *HER2* gene amplification on the endocrine responsiveness of the initial tumor cell population rather than an acquired property of a subpopulation of estrogen-independent tumor cells that selectively emerge after several months of therapy. The discordance between the data on tumor response and Ki67 changes raises several provocative questions. The first is a biologic issue. How can major responses to estrogen deprivation occur in ER-positive *HER2*-positive tumor despite poor, transient, or absent cytostatic effects? Second, which end point, Ki67 changes or tumor regression (or some combination of the two end points), will best predict the likelihood that the patient will do well on long-term adjuvant endocrine treatment?

Estrogen deprivation–induced tumor regression must be a complicated event, presumably involving not just the induction of cell cycle arrest, but also the involvement of other processes such as apoptosis, normalization of the tumor vasculature,[13] and other changes in the composition of the tumor mass. Certainly, there are considerable data that tumor angiogenesis can be estrogen dependent, perhaps through direct regulation of vascular endothelial growth factor,[14,15] and letrozole therapy can reduce tumor gadolinium contrast accumulation on breast magnetic resonance imaging, implying an effect of estrogen deprivation on the tumor vasculature.[11] With respect to apoptosis, this has been quite difficult to assess in neoadjuvant endocrine studies because cells staining for apoptosis markers are rare at baseline and not obviously modulated by endocrine therapy.[16] We have

Table 7. Cell Cycle CR (Ki67 = 1% or less in post-treatment sample) by *HER2* FISH Status in Letrozole-Treated Patients

Response	No. of *HER2* FISH-Positive Patients	No. of *HER2* FISH-Negative Patients	Total No.	Fisher *P*
Cell cycle CR				
Yes	2	111	113	
No	15	73	88	
Total	17	184	201	.0001

Abbreviations: CR, complete response; *HER2*, human epidermal growth factor receptor 2; FISH, fluorescence in situ hybridization.

Table 8. Cell Cycle CR (Ki67 = 1% or less in post-treatment sample) by *HER2* FISH Status in All Patients in the Study Whether Treated With Letrozole or Tamoxifen

Response	No. of *HER2* FISH-Positive Patients	No. of *HER2* FISH-Negative Patients	Total No.	Fisher *P*
Cell cycle CR				
Yes	3	148	151	
No	23	123	146	
Total	26	271	297	.0001

Abbreviations: CR, complete response; *HER2*, human epidermal growth factor receptor 2; FISH, fluorescence in situ hybridization.

presented preliminary data on tumor grade and have uncovered potential differences in the morphologic response to tamoxifen and letrozole. Specifically, letrozole seems to reduce nuclear grade to a greater extent than tamoxifen.[17] Whether these changes contribute to relative differences in treatment-induced decrease in tumor size is unclear.

The data presented in this article serve to emphasize a need to critically evaluate neoadjuvant endocrine therapy end points in relation to relapse risk. The few studies that have addressed this issue do support the notion that a neoadjuvant response, whether defined clinically or on the basis of Ki67, does portend a better prognosis.[18,19] Only when the relationships between short-term neoadjuvant outcomes and the risk of relapse are better understood will we be able to incorporate information on the short-term success of neoadjuvant endocrine therapy into planning other aspects of the adjuvant treatment plan, such as the need to administer chemotherapy or trastuzumab. Data on the influence of *HER2* status on the relative benefits of tamoxifen and letrozole as adjuvant therapy in the Breast International Group (BIG) 1-98 trial are, therefore, of considerable interest. A recent preliminary report indicated that *HER2*-positive status was associated with a significantly higher relapse rate in the BIG 1-98 trial, regardless of whether letrozole or tamoxifen was used.[20] This conclusion, if consistent in further follow-up, supports the validity of the Ki67-based predictive models presented in this article. An answer to the question of whether letrozole maintains a therapeutic advantage in the adjuvant setting despite *HER2* FISH-positive status (suggested by the clinical response data) also awaits longer follow-up of the BIG 1-98 trial population.

This investigation supports the notion that well-designed neoadjuvant endocrine therapy studies could be useful to identify novel molecular explanations for endocrine therapy resistance. The data outlined in Table 7 suggest that the majority of cases of endocrine resistance detected at the level of Ki67 remain unexplained because 73 (40%) of 184 ER-positive *HER2*-negative tumors failed to achieve a cell cycle CR and, therefore, display some degree of letrozole resistant-cell cycling. The combination of candidate gene sequencing, array-based comparative genomic hybridization, and gene expression profiling should allow the identification of these additional causes of endocrine therapy resistance and ultimately foster additional targeted approaches to the problem of endocrine therapy failure.

REFERENCES

1. Hu JC, Mokbel K: Does c-erbB2/HER2 overexpression predict adjuvant tamoxifen failure in patients with early breast cancer? Eur J Surg Oncol 27:335-337, 2001
2. Berry DA, Muss HB, Thor AD, et al: HER-2/neu and p53 expression versus tamoxifen resistance in estrogen receptor-positive, node-positive breast cancer. J Clin Oncol 18:3471-3479, 2000
3. Ellis MJ, Coop A, Singh B, et al: Letrozole is more effective neoadjuvant endocrine therapy than tamoxifen for ErbB-1- and/or ErbB-2-positive, estrogen receptor-positive primary breast cancer: Evidence from a phase III randomized trial. J Clin Oncol 19:3808-3816, 2001
4. Smith IE, Dowsett M, Ebbs SR, et al: Neoadjuvant treatment of postmenopausal breast cancer with anastrozole, tamoxifen, or both in combination: The Immediate Preoperative Anastrozole, Tamoxifen, or Combined With Tamoxifen (IMPACT) multicenter double-blind randomized trial. J Clin Oncol 23:5108-5116, 2005
5. Lal P, Tan LK, Chen B: Correlation of HER-2 status with estrogen and progesterone receptors and histologic features in 3,655 invasive breast carcinomas. Am J Clin Pathol 123:541-546, 2005
6. Ellis MJ, Coop A, Singh B, et al: Letrozole inhibits tumor proliferation more effectively than tamoxifen independent of HER1/2 expression status. Cancer Res 63:6523-6531, 2003
7. Dowsett M, Smith IE, Ebbs SR: Short-term changes in Ki-67 during neoadjuvant treatment of primary breast cancer with anastrozole or tamoxifen alone or combined correlate with recurrence-free survival. Clin Cancer Res 11:951s-958s, 2005
8. Eiermann W, Paepke S, Appfelstaedt J, et al: Preoperative treatment of postmenopausal breast cancer patients with letrozole: A randomized double-blind multicenter study. Ann Oncol 12:1527-1532, 2001
9. Dixon JM: Role of endocrine therapy in the neoadjuvant surgical setting. Ann Surg Oncol 11: 18S-23S, 2004
10. Going JJ, Keith WN, Neilson L, et al: Aberrant expression of minichromosome maintenance proteins 2 and 5, and Ki-67 in dysplastic squamous oesophageal epithelium and Barrett's mucosa. Gut 50:373-377, 2002
11. Tao Y, Klause A, Vickers A, et al: Clinical and biomarker endpoint analysis in neoadjuvant endocrine therapy trials. J Steroid Biochem Mol Biol 95:91-95, 2005
12. Dowsett M, Harper-Wynne C, Boeddinghaus I, et al: HER-2 amplification impedes the antiproliferative effects of hormone therapy in estrogen receptor-positive primary breast cancer. Cancer Res 61:8452-8458, 2001
13. Jain RK: Normalization of tumor vasculature: An emerging concept in antiangiogenic therapy. Science 307:58-62, 2005
14. Ruohola JK, Valve EM, Karkkainen MJ, et al: Vascular endothelial growth factors are differentially regulated by steroid hormones and antiestrogens in breast cancer cells. Mol Cell Endocrinol 149:29-40, 1999
15. Heer K, Kumar H, Read JR, et al: Serum vascular endothelial growth factor in breast cancer: Its relation with cancer type and estrogen receptor status. Clin Cancer Res 7:3491-3494, 2001
16. Dowsett M, Ebbs SR, Dixon JM, et al: Biomarker changes during neoadjuvant anastrozole, tamoxifen, or the combination: Influence of hormonal status and HER-2 in breast cancer—A study from the IMPACT trialists. J Clin Oncol 23:2477-2492, 2005
17. Turner J, Bentey R, Tao Y, et al: Changes in tumor grade with neoadjuvant endocrine therapy: Morphological evidence for fundamental differences in the anti-tumor effects of letrozole and tamoxifen. Breast Cancer Res Treat 88:S37, 2004 (abstr 407)
18. Horobin JM, Preece PE, Dewar JA, et al: Long-term follow-up of elderly patients with locoregional breast cancer treated with tamoxifen only. Br J Surg 78:213-217, 1991
19. Miller WR, Anderson TJ, White S, et al: Growth factor signaling in clinical breast cancer and its impact on response to conventional therapies: The Edinburgh experience. Endocr Relat Cancer 12:S119-S123, 2005 (suppl 1)
20. Viale G, Regan M, Dell'Orto P, et al: Central review of ER, PgR and HER-2 in BIG 1-98 evaluating letrozole vs. tamoxifen as adjuvant endocrine therapy for postmenopausal women with receptor-positive breast cancer. Presented at the 28th San Antonio Breast Cancer Symposium, San Antonio, TX, December 8-11, 2005 (abstr 44)

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Neoadjuvant endocrine therapy models.

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Neoadjuvant therapy is therapy administered before surgical intervention and while the tumor remains in the breast. It may be given to treat large, locally advanced tumors, with the aim of shrinking them and thus making their surgical excision either simply possible or less radical. Most neoadjuvant therapy is chemotherapy, but adjuvant endocrine therapy is increasingly used in hormone-sensitive tumors; for example, those responsive to tamoxifen. Repeat biopsies aimed at assessing response to treatment--for example, by examining estrogen receptor status or markers of proliferation in tumor tissue--may be taken during the course of adjuvant therapy. In this chapter, the essential protocols associated with designing neoadjuvant trials are described, methods of assessing response to neoadjuvant therapy are detailed, and various approaches to collecting appropriate clinical samples and their assessment are presented.

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ull Paper

roliferation, steroid receptors and clinical/pathological response n breast cancer treated with letrozole

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Sixty-three postmenopausal women with large primary breast cancers were treated with neoadjuvant letrozole (2.5 mg daily) for 3 months. Tumour samples were taken at diagnosis and after 10–14 days and 3 months treatment. Immunohistochemical staining for Ki67, oestrogen receptor (ER) and progesterone receptor (PgR) was performed and related to clinical (ClinR) and pathological responses (PathR) after 3 months treatment. ClinR was observed in 48 of 63 cases (76.2%) and PathR in 47 of 62 (75.8%). Pretreatment Ki67 scores were similar in responders (R) and non-responders (NR). Highly significant Ki67 decreases occurred in all tumour subgroups at 10–14 days ($P<0.005$). A significant difference in Ki67 scores at 10–14 days ($P<0.007$) was found between PathR and PathNR but not between ClinR and ClinNR. At 3 months, decreases from pretreatment Ki67 scores were highly significant in all tumour subgroups irrespective of response status. However, whereas Ki67 scores were significantly different between pathological R and NR ($P=0.009$), the corresponding comparison of ClinR status was not. Significant decreases between 10–14 days and 3 months were found only in ClinR and PathR ($P=0.02$ and 0.045, respectively). Treatment significantly reduced PgR expression at 14 days and 3 months (both $P<0.0001$), but the level of changes was not different between response status groups. In summary, letrozole produces rapid and profound decreases in expression of Ki67 and PgR but changes do not always correlate with clinical and pathological responses.

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Keywords: breast cancer; neoadjuvant therapy; aromatase inhibitor; proliferation; steroid receptors; clinical response

etrozole is a third-generation aromatase inhibitor, which in inical trials has been shown to be highly effective in post-ıenopausal women with oestrogen receptor (ER)-positive breast ıncer (Eiermann *et al*, 2001; Mouridsen *et al*, 2001; Goss *et al*, 003; Rose *et al*, 2003). Previous studies using neoadjuvant therapy ave shown that letrozole may produce profound changes in ımour pathology and immunohistochemical markers (Dixon *t al*, 2001; Ellis *et al*, 2001, 2003; Miller *et al*, 2003; Anderson *et al*, 004). Furthermore, it is clear that the clinical effects of eoadjuvant treatment with third-generation aromatase inhibitors ı postmenopausal women are not dissimilar to those seen with eoadjuvant chemotherapy (Dixon *et al*, 2001) and are achieved ·ith less morbidity. Comparative studies have also shown that the ffects of third-generation aromatase inhibitors are more consis-ːnt and greater than tamoxifen on proliferation (as measured by .i67) and markers of oestrogen action (progesterone receptors PgR) and trefoil factor 1) (Ellis *et al*, 2003; Miller *et al*, 2003; .nderson *et al*, 2004). It is therefore interesting that in the eoadjuvant setting, letrozole yields significantly superior clinical esults than tamoxifen and also appears to be more effective in articular subgroups such as tumours with low ER levels and verexpression of HER-2 (Ellis *et al*, 2001). However, the timescale f these effects and their relationship to clinical and pathological response as assessed at the end of treatment has yet to be fully defined. The aim of the present study was therefore to assess the effects of letrozole on the proliferation marker Ki67 and receptors for oestrogen and progesterone by immunohistochemical assessment in serial biopsies from primary breast cancers taken before, at 10–14 days and at 3 months into treatment.

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MATERIALS AND METHODS

Patients

A total of 63 postmenopausal women presented to the Edinburgh Breast Unit with large (>3 cm) primary breast cancer, which were ER-rich (Allred score 5–8). (However, review of the cases in the research laboratory showed that all patients recruited to the study had ER scores of 7 or 8.) All patients apart from 12 were technically operable. The primary clinical objective was to downstage tumours such that those who were inoperable became amenable for surgery and those who would have required mastectomy could become candidates for breast conservation. This series represents consecutive patients recruited but excluding cases in which the tumour was shown to be multifocal or of special histological type (e.g. mucinous, tubular/cribriform and lobular). All patients gave informed consent to be included in the study, which had been approved by the local ethics committee (2001/W/BU/09 and 2001/W/BU/10).

Treatment

All patients received letrozole (2.5 mg, daily) for 3 months. Serial measurements of the primary tumours were taken before, at 6 weeks and at 3 months by calliper and ultrasound as described previously (Forouhi *et al*, 1994; Dixon, 2001). The tumour was also imaged mammographically before and at 3 months. Core biopsies were taken at the start, after 10–14 days and at 3 months of treatment as described previously (Iqbal *et al*, 2002). All patients, apart from eight patients (who electively continued on treatment), received definitive surgery at 3 months.

Response assessment

Tumour volumes were determined from ulstrasound measurements as described by Forouhi *et al* (1994). Reduction in volume over a 3 month period >50% was regarded as clinical response; this includes both complete and partial responders.

Pathological response was determined by comparing biopsies taken before and after 3 months of treatment. Only marked reduction in cellularity and/or a clear increase in fibrosis were used as evidence of pathological response. Although these changes are essentially subjective, they were confirmed by two observers working independently. The criteria may underestimate actual morphological changes occurring to a lesser degree, but these were excluded because of the difficulty in comparing histological appearances on limited tissue such as in core biopsies with those in the more substantial material obtained at tumour excision. No case was classified as a complete PathR, residual evidence of malignant cells being evident.

Immunohistochemistry

Immunohistochemistry staining with antibody to MIB1 (Ki-67) antigen (Europath Ltd, Cornwall, UK) diluted ×50 was used as a measure of tumour cell proliferation. Reactivity was detected by an ABC–peroxidase–antiperoxidase (PAP) method, and scored according to the method described by Going (1994). A change of >40% between different paired biopsies was taken as being meaningful (Ellis *et al*, 1998; Iqbal *et al*, 2002) and a value of <1% was regarded as indicating a lack of proliferation.

Reactivity for ER or PgR was performed by the PAP method, after microwave antigen retrieval, using ERα antibody clone 6f11/2 (Novocastra Ltd, Newcastle, UK) and PgR antibody clone PgR636 (DAKO Labs, Ely, UK) using the DAKO EnVision system according to the manufacturer's instruction. Results were scored on a scale of 0–3 for staining intensity (with each successive score denoting increasing intensity), and on a score of 0–5 for increasing proportion of positive cancer nuclei (0 = none, 1 = <1%, 2 = 1–10%, 3 = 11–33%, 4 = 34–66%, 5 = >66%). The values were then summed into a category score within a range of 0–8 (Allred *et al*, 1998).

Statistics

Non-parametric comparisons using either Wilcoxon rank or Spearman paired testing was employed and, where appropriate, 3×2 χ^2 testing.

RESULTS

Clinical and pathological response

Of the 63 patients, 48 (76.2%) were classified as clinical responders (ClinR). With regard to pathological response, one case was not assessable because of insufficient material after biopsy at 3 months. Of the remaining 62 cases, 47 (75.8%) had clear evidence of pathological response. Although response rates were similar, there was not exact concordance between clinical and pathological outcomes. Thus, 42 of 48 ClinR were also pathological responders (PathR) (six were pathological non-responders (PathNR)) and nine of 15 clinical non-responders (ClinNR) were also PathNR. Of the remaining six cases, five were PathR and one case was pathologically not assessable.

Proliferation (Ki67)

The tumour Ki67 scores for pretreatment, 10–14 days and 3 months samples, subdivided according to clinical and pathological response status, are shown in Table 1.

No significant differences were apparent for pretreatment group scores between responders (R) and non-responders (NR) whether assessed clinically or pathologically. However, at 10–14 days, whereas there was no difference between ClinR and ClinNR, the PathNR were significantly higher than PathR ($P = 0.024$). Similarly at 3 months, although there was no significant difference between ClinR and ClinNR, values in PathNR were significantly higher than in PathR ($P = 0.009$).

With regard to group changes in Ki67 with treatment, the scores at 10–14 days and 3 months were highly significantly decreased, as compared with those in paired pretreatment biopsies. These changes were irrespective of clinical or pathological response status. In terms of changes occurring between 10–14 days and 3 months, a significant decrease was seen in ClinR ($P = 0.02$) and PathR ($P = 0.045$). However, values were not significantly different between 10–14 days and 3 months in ClinNR and PathNR.

Changes in Ki67 with treatment in individual tumours are summarised in Table 2. In terms of >40% change criteria for ClinR, 52 of 63 cases (82.5%) showed a decrease at 10–14 days, and 53 of 63 tumours (84%) showed a reduction at 3 months. These reductions were seen in both ClinR and ClinNR groups, and there was no significant difference between them. However, for pathological assessments, there was a significant difference between PathR and PathNR at 10–14 days ($P = 0.034$) but not at 3 months. Treatment effects were also assessed on the basis of reducing Ki67 scores to <1%. It can be seen that whereas 11 cases (17.5%) were reduced to <1% by 10–14 days, these numbers markedly increased to 27 (42.9%) by 3 months. Whereas the reduction to <1% was seen in both responding and non-responding tumours, the increase in numbers between 10–14 days and 3 months is predominantly associated with responding tumours.

Different patterns of changes in Ki67 over the treatment period could be detected. These are shown in Figure 1. The largest cohort of tumours (47) showed substantial decreases at 10–14 days, which were maintained or fell further at 3 months (in these tumours, changes in Ki67 at 10–14 days were predictive of those at 3 months). However, there was a small cohort of five patients in which a decrease at 10–14 days was followed by a substantial rise

Table 1 Ki67 scores in tumour taken before and after 10–14 days and 3 months of treatment, subdivided according to response status

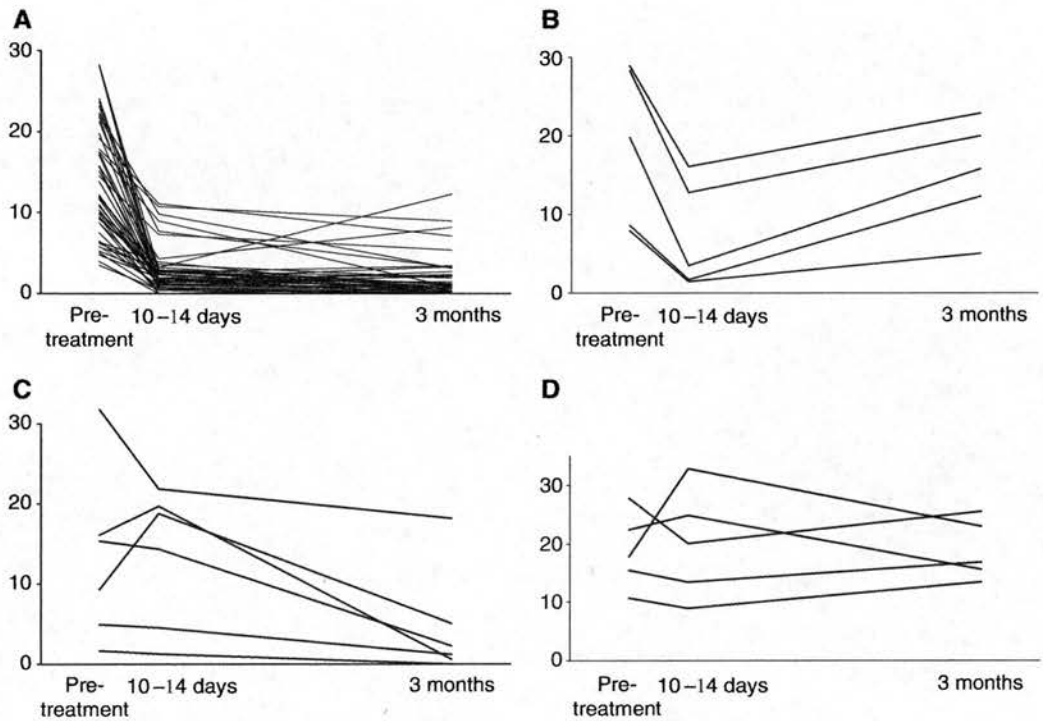
	Ki67 scores (mean ± s.e.m.)		
	Pretreatment	**10–14 days**	**3 months**
Clinical responders (48)	14.17 ± 1.10	5.11 ± 0.98^{+}	4.13 ± 0.96$^{+,\blacklozenge}$
Clinical non-responders (15)	15.29 ± 2.06	6.72 ± 2.04^{+}	5.85 ± 1.91$^{*,\circ}$
	$P = 0.70$	$P = 0.77$	$P = 0.13$
Pathological responders (47)	14.03 ± 1.09	4.02 ± 085^{+}	3.47 ± 0.90$^{+,\blacklozenge\blacklozenge}$
Pathological non-responders (15)	15.97 ± 2.22	9.35 ± 2.22**	8.11 ± 2.01$^{***,\circ}$
	$P = 0.52$	$P = 0.024$	$P = 0.009$

Compared with pretreatment tumour: $^{+}P < 0.0001$; $^{*}P = 0.003$; $^{**}P = 0.007$; $^{***}P = 0.009$. Compared with tumour taken after 10–14 days of treatment: $^{\blacklozenge}P = 0.02$; $^{\blacklozenge\blacklozenge}P = 0.045$: $^{\circ}P$ = NS.

able 2 Changes in tumour Ki67 scores with treatment

	No. of patients							
	Changes at 10–14 days				Changes at 3 months			
	Increase	No change	Decrease		Increase	No change	Decrease	
			by 40%	to <1%			by 40%	<1%
inical responders (48)	1	6	41	(8)	0	7	41	(23)
inical non-responders (15)	1	3	11	(3)	1	2	12	(4)
otal	2	9	52	(11)	1	9	53	(27)
			$P=0.49$				$P=0.20$	
thology responders (47)	1	4	42	(9)	0	6	41	(23)
thology non-responders (15)	1	5	9	(2)	1	3	11	(3)
otal	2	9	51	(11)	1	9	52	(26)
			$P=0.034$				$P=0.15$	

atistical comparison of increase (>40%), decrease (>40%) or no change (<40%) compared with pretreatment values by 3 × 2 chi-square testing.



igure 1 Tumour Ki67 scores before and after 10–14 days and 3 months treatment with letrozole. (**A**) Cases that show decreases (>40%) at both 10–4 days and 3 months. (**B**) Cases that show decreases (>40%) at 10–14 days but not at 3 months. (**C**) Cases that show no change at 10–14 days but a ecrease (>40%) at 3 months. (**D**) Cases that show no decrease at either 10–14 days or 3 months.

n score at 3 months (in these tumours, changes in Ki67 at 10–14 ays did not therefore concur with those at 3 months). Of the 11 hat did not decrease at 10–14 days, six decreased at 3 months Ki67 changes therefore did not concur at 10–14 days and 3 nonths) and five were not reduced at 3 months (lack of change in Ki67 at 10–14 days was predictive of no change at 3 months). hese patterns of Ki67 did not correlate with ClinR and PathR henotype (Figure 2).

Progesterone receptors

Of the 63 cases, 57 (90%) were PgR + ve; of the six negatives, five were ClinR and four were PathR. The changes with treatment of PgR staining subdivided according to ClinR and PathR status are summarised in Table 3.

Treatment reduced PgR scores such that, at 10–14 days, values were significantly lower than those in the paired pretreatment biopsy ($P<0.0001$). This decrease was found in all tumour subgroups irrespective of clinical or pathological assessment status. Similar highly statistically significant decreases were also seen when pretreatment values were compared with corresponding pairs at 3 months ($P<0.0001$). Of note is the high proportion of positive tumours that decreased to 0 by 10–14 days (45.6%). This percentage rose to 66.7% at 3 months. A comparison of 10- to 14-day biopsies with those at 3 months showed significant decreases with extended treatment with the groups of ClinR and PathR, whereas no significant difference was detected in the NR group

(but this represents a small number of pairs). In terms of comparisons between tumours R or NR assessed either clinically or pathologically, no significant differences were detected either at pretreatment, 10–14 days or 3 months in absolute values (data not shown).

Oestrogen receptors

There was no significant difference in ER score pretreatment, after 10–14 days and after 3 months. Neither was there a significant change with treatment with all biopsies scoring 7 or 8 throughout (data not shown).

DISCUSSION

The observation that neoadjuvant treatment with letrozole is associated with a marked reduction in the immunohistochemical expression of Ki67 and PgR confirms our previous findings (Miller *et al*, 2003; Anderson *et al*, 2004) and those of others (Ellis *et al*, 2001, 2003). However, the present study extends our previous work by demonstrating that such effects are evident as early as 10–14 days into treatment in over 80% of cases. Similar results have recently been presented for anastrozole (Dowsett *et al*, 2005a, b). The same group have also presented results from a randomised neoadjuvant trial comparing the aromatase inhibitor vorozole with tamoxifen. Ki67 levels fell within 2 weeks of treatment and remained suppressed at surgery 3 months later (Harper-Wynne *et al*, 2002). These effects are therefore apparent before evidence of morphological changes in tumour pathology and clinical evidence of changes in tumour volume. It was of interest in the present paper to determine whether changes in proliferation and PgR expression related to and/or predicted for subsequent ClinR and PathR.

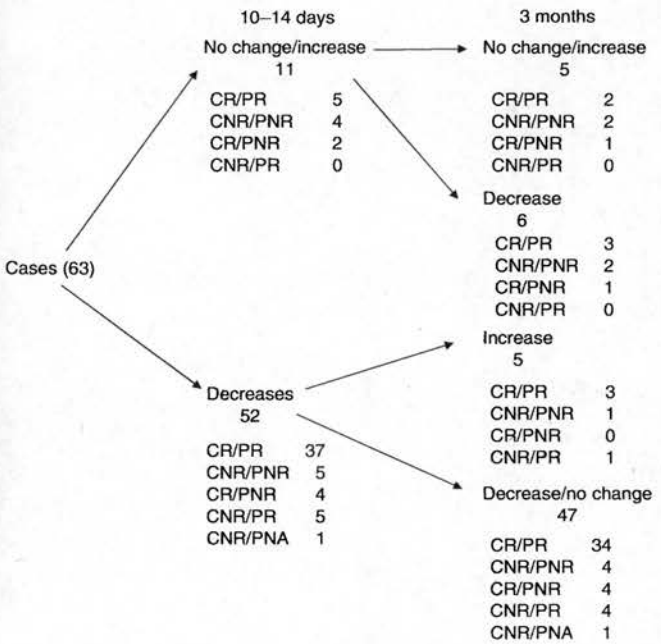


Figure 2 Flow diagram indicating number of cases grouped according to Ki67 changes at 10–14 days and 3 months and further subdivided according to final clinical/pathological response.

In terms of assessing proliferation status with Ki-67 scores, we have analysed results in three ways: (i) comparison of tumour scores at individual time points grouped according to response status at 3 months, (ii) classifying a >40% change in Ki-67 between different time points as evidence of a meaningful change in proliferation and (iii) comparing the number of cases in which proliferation is reduced to <1%, a value that we have regarded as a state of virtual non-proliferation. By using these multiple analyses, we hoped to derive impressions not only of group trends but effects and degree in individual cases.

Group comparisons of mean Ki-67 scores at individual study time points revealed interesting differences according to whether response was assessed clinically or pathologically. Thus, the only detectable significant differences were in groups subdivided by pathological assessment in which higher mean scores were found in non-responding tumours at both 10–14 days and 3 months. Interestingly, the same general pattern was evident when categorising individual cases according to >40% reduction (the only significant difference was seen between PathR and PathNR at 10–14 days). The restriction of significant effects to pathological assessment probably reflects the closer association between two histological assessments rather than that between histology and tumour size. It is also worth noting that the tumour morphology after treatment will be determined by factors in addition to proliferation, such as cell loss. In this respect, although Ki67 is a primary marker of proliferation, it can also be a secondary reflection of cell death (Archer *et al*, 2003).

A reduction of >40% in Ki-67 was apparent in most cases at 10–14 days, and extended treatment to 3 months was associated with only minor changes in the proportion of tumours that displayed a >40% decrease in proliferation. This is in contrast to the results based upon the more profound criteria of a decrease to an absolute value of <1%. These results show that, remarkably, even after 10–14 days of treatment, 17% of tumours have reached this state of virtual non-proliferation, and this was irrespective of whether tumours subsequently displayed evidence of clinical or pathological response. However, the proportion of cases falling to <1% proliferation increases further to 43% by 3 months. Interestingly, this incremental effect with time appears restricted to those tumours that had either a pathological or clinical response

Table 3 Changes in tumour PgR score with treatment

	No. of patients							
	Changes at 10–14 days				Changes at 3 months			
	Increase	No change	Decrease	(Decrease to 0)	Increase	No change	Decrease	(Decrease to 0)
Clinical responders (48)	1	11	36	(18)	0	9	39	(29)
Clinical non-responders (15)	0	2	13	(8)	0	2	13	(9)
Pathology responders (47)	1	11	35	(16)	0	9	38	(26)
Pathology non-responders (15)	0	2	13	(9)	0	2	13	(11)

PgR = progesterone receptor.

atus at 3 months. It is clear that letrozole is capable of producing creased suppression of proliferation when used over an extended riod.

The overall perspective therefore is that letrozole is capable of oducing a rapid reduction in tumour proliferation that is seen in ost tumours irrespective of subsequent clinical and pathological sponse, but that incremental effects on proliferation (as onitored by scores of $<1\%$) are additionally seen in the period tween 10–14 days and 3 months, and these are largely restricted PathR or ClinR.

Whereas changes in Ki-67 levels have been revealed by group mparisons, the strength of neoadjuvant studies is that it is ssible to examine differences in individual cases and classify mours according to sequential changes in proliferation. Thus, nsistent with the general trends discussed above, most tumours splayed a substantial decrease in proliferation $>40\%$ by 10–14 ys and this was sustained at 3 months. However, it was also ssible to identify (i) a small cohort that initially had decreased oliferation at 10–14 days but which largely disappeared by 3 onths, (ii) tumours that failed to demonstrate a decrease in oliferation at 10–14 days but had a delayed decrease apparent 3 months and (iii) cases that failed to display a decrease in oliferation at both 10–14 days and 3 months. It is therefore portant not only to discuss relationships between clinical/ thological response and proliferation at individual time points, t also to take into account the patterns of change in response to eatment.

Statistically significant differences were detected between PathR d PathNR in (i) group levels of Ki67 at 10–14 days and (ii) the roportion of cases decreasing in Ki67 $>40\%$ between pretreatent and 10–14 days. However, there was a large overlap in values 10–14 days between PathR and PathNR, and individual tumours uld display an increase, no change or a decrease in Ki67 respective of being PathR or PathNR. Consequently, measurements of Ki67 in individual cases do not accurately predict for bsequent pathological (or clinical) response. A similar lack of rediction between Ki67 changes and clinical response to nastrozole has been observed in the recently reported IMPACT eoadjuvant trial (Dowsett *et al*, 2005a) (although the short-term hanges in proliferation did parallel recurrence-free survival etween the three treatment groups, anastrozole, tamoxifen and rimidex combined with tamoxifen) (Dowsett *et al*, 2005b). As a onsequence, consideration therefore needs to be given as to why lear decreases in cellular proliferation at 10–14 days do not anslate into pathological response and why conversely respondng cases can show no change or even an increase in Ki67 with eatment.

It is possible that lack of correlation in some cases relates in part imprecise measurements of proliferation or misclassification of esponse. In terms of immunohistochemical assessment of Ki67, e have already published data on reproducibility in breast cancer iopsies (Iqbal *et al*, 2002). These showed that, because of inherent eterogeneity, marked variation in Ki67 score may be observed in he same tumour without intervening treatment. However, this is estricted to occasional tumours, and the number of cases in the resent study with discordance between proliferation changes and linical/pathological response is greater than would have been xpected. Furthermore, in order to reduce spurious results, we ave used three different criteria for assessing changes in Ki67. In erms of the impact of assessment of clinical response, potential ources of inconsistencies have been considered elsewhere and ary according to the technology employed (Forouhi *et al*, 1994). n the present studies, clinical responses were based primarily pon ultrasound measurements, but they were substantiated by arallel calliper and mammographic measurements in all cases. or ease of presentation, clinical response was dichotomised and it s possible that the use of continuous variables might have been nore informative. However, preliminary analyses using continuous variables did not reveal better relationships between proliferation and response (data not shown). There are also limitations to the assessment of pathological response in that the pretreatment assessments (and some of the post-treatment) were performed on core biopsies, which are not guaranteed to be representative of the total tumour mass. It is also possible that assessment of ClinR/PathR at the single time point of 3 months is associated with chronological inaccuracy in that certain tumours classified as NR may go on to respond with extended treatment (Dixon *et al*, 2005). There is no doubt that response is not complete by 3 months and treatment up to 12 months may be associated with (a) further tumour shrinkage and (b) an increased incidence of complete ClinR.

Although methodological imprecision might be influential in some cases, it is unlikely that these totally account for the lack of association between proliferation and response. Other reasons need to be considered including the possibility that reduction in proliferation alone may not produce tumour shrinkage and cell death or apoptosis may be equally influential. Although we have not measured apoptosis in tumour samples from the present study (because assessment in core biopsies was not sufficiently reproducible), we did not find apoptosis to be predictive of response in other tumour samples from patients offered neoadjuvant endocrine therapy (Anderson *et al*, 2002). However, this may be because differences are small and transient; primary effects on apoptosis may also be masked by those occurring secondarily (e.g. as a result of decreased proliferation).

Another cause for a reduction in proliferation at 10–14 days not translating into subsequent tumour response could be that the effect is transient and not maintained over a sufficiently extended period to produce tumour shrinkage. However, in the present study, four of the five non-responding tumours with a reduction in Ki67 had a sustained decrease in proliferation to 3 months. A disconnect between changes in proliferation as observed at 10–14 days with subsequent clinical/pathological response could also be explained if the proliferation response was delayed. Interestingly, six of 11 tumours showed a delayed reduction in Ki67 scores; three of these were classified as PathR/ClinR.

It is worth noting that in 11 of 63 (17%) cases, change in proliferation at 10–14 days failed to predict that at 3 months. Furthermore, most importantly, change in proliferation at 10–14 days failed to predict clinical response in 18 out of 63 (29%) cases and pathological response in 14 out of 62 (23%) tumours. Change in proliferation at 10–14 days is therefore not an accurate surrogate of clinical/pathological response at 3 months.

The other marker that showed major changes with therapy was PgR. Thus, 78% of cases displayed a reduction of at least 1 category score by 10–14 days and was maintained to 3 months. Reduction occurred irrespective of subsequent clinical/pathological response. The extent of effect may be gauged by the percentage of cases reduced to negativity (40% at 10–14 days and 60% at 3 months); again the decrease to negativity was irrespective of clinical or pathological response. As PgR is a marker of signalling from the ER, these changes are clear evidence of the anti-oestrogenic effects of letrozole and contrast with those of tamoxifen (Anderson *et al*, 2002; Miller *et al*, 2003).

Although we have not formally presented the correlations between changes in PgR and proliferation, there was a positive relationship between them. However, there were instances of discordance whereby at both 10–14 days and 3 months, tumours displayed either a phenotype of reduced proliferation but stable PgR or unchanged proliferation and reduced PgR. As reduced PgR expression is a marker of oestrogen deprivation, it is unlikely that the lack of effect on Ki67/ClinR/PathR in these cases is because of lack of aromatase inhibition. Hence, changes in PgR with treatment, although marked and occurring early, are not predictive of clinical or pathological response. It was also of interest to examine changes in PgR score in tumours that initially displayed a

decrease in Ki67 at 10–14 days but an increase at 3 months. This phenotype could reflect adaptive changes leading to an oestrogenic stimulation or a state of hypersensitivity to oestrogen (Santen *et al*, 2004); however, changes in PgR in this cohort of five tumours with this phenotype revealed three cases that decreased between 10–14 days and 3 months and two cases that were negative at all time points, providing no evidence for adaptive changes or hypersensitivity to a reduced oestrogenic environment.

In conclusion, the present study has provided evidence that neoadjuvant letrozole produces marked effects on levels of Ki67 and PgR within 10–14 days. Although early changes in proliferation are less likely to occur in tumours that show no pathological response at 3 months, the effects can be seen irrespective of clinical and/or pathological response in individual cases. Ki67 scores and PgR expression are therefore of limited value as predictors of response. They do however reflect the potent anti-oestrogenic and anti-proliferative properties of the third-generation aromatase inhibitors and it has been suggested that such changes may relate to long-term outcome (Ellis *et al*, 2003; Dowsett *et al*, 2005b).

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REFERENCES

Allred DC, Harvey JM, Berardo M, Clark GM (1998) Prognostic and predictive factors in breast cancer by immunohistochemical analysis. *Mod Pathol* **11**: 155–168

Anderson TJ, Dixon JM, Murray J, Renshaw L, White S, Miller WR (2004) Early changes in tumour Ki67 expression differentiate for pathological (but not clinical) response in breast cancers treated neoadjuvantly with letrozole. *Breast Cancer Res Treat* **88**(Suppl 1): S50

Anderson TJ, Dixon JM, Stuart M, Sahmoud T, Miller WR (2002) Effect of neoadjuvant treatment with anastrozole on tumour histology in postmenopausal women with large operable breast cancer. *Br J Cancer* **87**: 334–338

Archer CD, Parton M, Smith IE, Ellis PA, Salter J, Ashley S, Gui G, Sacks N, Ebbs SR, Allum W, Nasiri N, Dowsett M (2003) Early changes in apoptosis and proliferation following primary chemotherapy for breast cancer. *Br J Cancer* **89**: 1035–1041

Dixon JM (2001) Neoadjuvant endocrine therapy. In *Aromatase Inhibition and Breast Cancer*, Miller WR, Santen RJ (eds). pp 103–116. UK: Pub Marcel Dekker

Dixon JM, Love CD, Bellamy CO, Cameron DA, Leonard RC, Smith H, Miller WR (2001) Letrozole as primary medical therapy for locally advanced and large operable breast cancer. *Breast Cancer Res Treat* **66**(3): 191–199

Dixon JM, Renshaw L, Murray J, Macaskill EJ, Young O, Miller WR (2005) Surgical issues surrounding use of aromatase inhibitors. *J Steroid Biochem Mol Biol* **95**: 97–103

Dowsett M, Ebbs SR, Dixon JM, Skene A, Griffith C, Boeddinghaus I, Salter J, Detre S, Hills M, Ashley S, Francis S, Walsh G, Smith IE (2005a) Biomarker changes during neoadjuvant anastrozole, tamoxifen, or the combination: influence of hormonal status and HER-2 in breast cancer – a study from the IMPACT trialists. *J Clin Oncol* **23**(11): 2477–2492

Dowsett M, Smith IE, Ebbs SR, Dixon JM, Skene A, Griffith C, Boeddinghaus I, Salter J, Detre S, Hills M, Ashley S, Francis S, Walsh G, on behalf of the IMPACT Trialists (2005b) Short-term changes in Ki67 during neoadjuvant treatment of primary breast cancer with anastrozole or tamoxifen alone or combined correlate with recurrence-free survival. *Clin Cancer Res* **11**(Suppl 1): 951s–958s

Eiermann W, Paepke S, Appfelstaedt J, Llombart-Cussac A, Eremin J, Vinholes J, Mauriac L, Ellis M, Lassus M, Chaudri-Ross HA, Dugan M, Borgs M, Letrozole Neo-Adjuvant Breast Cancer Study Group (2001) Preoperative treatment of postmenopausal breast cancer patients with letrozole: a randomized double-blind multicenter study. *Ann Oncol* **12**(11): 1527–1532

Ellis MJ, Coop A, Singh B, Mauriac L, Llombert-Cussac A, Janicke F, Miller WR, Evans DB, Dugan M, Brady C, Quebe-Fehling E, Borgs M (2001) Letrozole is more effective neoadjuvant endocrine therapy than tamoxifen for ErbB-1- and/or ErbB-2-positive, estrogen receptor-positive primary breast cancer: evidence from a phase III randomized trial. *J Clin Oncol* **19**(18): 3808–3816

Ellis MJ, Coop A, Singh B, Tao Y, Llombart-Cussac A, Janicke F, Mauriac L, Quebe-Fehling E, Chaudri-Ross HA, Evans DB, Miller WR (2003) Letrozole inhibits tumor proliferation more effectively than tamoxifen independent of HER1/2 expression status. *Cancer Res* **63**(19): 6523–6531

Ellis PA, Smith IE, Detre S, Burton SA, Salter J, A'Hern R, Walsh G, Johnston SR, Dowsett M (1998) Reduced apoptosis and proliferation and increased Bcl-2 in residual breast cancer following preoperative chemotherapy. *Breast Cancer Res Treat* **48**(2): 107–116

Forouhi P, Walsh JS, Anderson TJ, Chetty U (1994) Ultrasonography as a method of measuring breast tumour size and monitoring response to primary systemic treatment. *Br J Surg* **81**(2): 223–225

Going JJ (1994) Efficiently estimated histologic cell counts. *Hum Pathol* **4**: 333–336

Goss PE, Ingle JN, Martino S, Robert NJ, Muss HB, Piccart MJ, Castiglione M, Tu D, Shepherd LE, Pritchard KI, Livingston RB, Davidson NE, Norton L, Perez EA, Abrams JS, Therasse P, Palmer MJ, Pater JL (2003) A randomized trial of letrozole in postmenopausal women after five years of tamoxifen therapy for early-stage breast cancer. *N Engl J Med* **349**(19): 1793–1802

Harper-Wynne CL, Sacks NP, Shenton K, MacNeill FA, Sauven P, Laidlaw IJ, Rayter Z, Miall S, Howes A, Salter J, Hills MJ, Lowe FM, A'Hern R, Nasiri N, Doody D, Iqbal J, Dowsett M (2002) Comparison of the systemic and intratumoural effects of tamoxifen and the aromatase inhibitor vorozole in postmenopausal patients with primary breast cancer. *J Clin Oncol* **20**(4): 1026–1035

Iqbal S, Anderson TJ, Marson LP, Prescott RJ, Dixon JM, Miller WR (2002) MIB-1 assessments in breast cancers. *Breast* **11**(3): 252–256

Miller WR, Dixon JM, Macfarlane L, Cameron D, Anderson TJ (2003) Pathological features of breast cancer response following neoadjuvant treatment with either letrozole or tamoxifen. *Eur J Cancer* **39**(4): 462–468

Mouridsen H, Gershanovich M, Sun Y, Perez-Carrion R, Boni C, Monnier A, Apffelstaedt J, Smith R, Sleeboom HP, Janicke F, Pluzanska A, Dank M, Becquart D, Bapsy PP, Salminen E, Snyder R, Lassus M, Verbeek JA, Staffler B, Chaudri-Ross HA, Dugan M (2001) Superior efficacy of letrozole *vs* tamoxifen as first-line therapy for postmenopausal women with advanced breast cancer: results of a phase III study of the International Letrozole Breast Cancer Group. *J Clin Oncol* **19**: 2596–2606

Rose C, Vtoraya O, Pluzanska A, Davidson N, Gershanovich M, Thomas R, Johnson S, Caicedo JJ, Gervasio H, Manikhas G, Ben Ayed F, Burdette-Radoux S, Chaudri-Ross HA, Lang R (2003) An open randomised trial of second-line endocrine therapy in advanced breast cancer: comparison of the aromatase inhibitors letrozole and anastrozole. *Eur J Cancer* **39**(16): 2318–2327

Santen RJ, Song RX, Zhang Z, Yue W, Kumar R (2004) Adaptive hypersensitivity to estrogen: mechanism for sequential responses to hormonal therapy in breast cancer. *Clin Cancer Res* **10**(1 Part 2): 337S–345S

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Aromatase inhibitors: Cellular and molecular effects☆

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Abstract

Marked cellular and molecular changes may occur in breast cancers following treatment of postmenopausal breast cancer patients with aromatase inhibitors. Neoadjuvant protocols, in which treatment is given with the primary tumour still within the breast, are particularly illuminating. In Edinburgh, we have shown that 3 months treatment with either anastrozole, exemestane or letrozole produces pathological responses in the majority of oestrogen receptor (ER)-rich tumours (39/59) as manifested by reduced cellularity/increased fibrosis. Changes in histological grading may also take place, most notably a reduction in mitotic figures. This probably reflects an influence on proliferation as most tumours (82%) show a marked decrease in the proliferation marker, Ki67. These effects are generally more dramatic than seen with tamoxifen given in the same setting. Differences between aromatase inhibitors and tamoxifen are also apparent in changes in steroid hormone expression. Thus, immuno-staining for progesterone receptor (PgR) is reduced in almost all cases by aromatase inhibitors, becoming undetectable in many. This contrasts with effects of tamoxifen in which the most common change on PgR is to increase expression. Changes in proliferation occur rapidly following the onset of exposure to aromatase inhibitors. Thus, neoadjuvant studies with letrozole in which tumour was sampled before and after 14 days and 3 months treatment show that decreased expression of Ki67 occur at 14 days and, in many cases, the effect is greater at 14 days than 3 months. These early changes precede evidence of clinical response but do not predict for it. However, this study design has allowed RNA analysis of sequential biopsies taken during the neoadjuvant therapy. Based on clustering techniques, it has been possible to subdivide tumours into groups showing distinct patterns of molecular changes. These changes in tumour gene expression may allow definition of tumour cohorts with differing sensitivity to aromatase inhibitors and permit early recognition of response and resistance.

Keywords: Aromatase inhibitors; Pathology; Proliferation; Progesterone receptor; RNA analysis

1. Introduction

Recently, a new generation of inhibitors have been developed which block the aromatase enzyme with immense potency and exquisite specificity [1–7]. Consequently, they suppress aromatase activity and endogenous oestrogen levels in postmenopausal women more effectively than ever before. In translating these endocrine influences into cellular and molecular effects, many of the data presented in this review are derived from patients given neoadjuvant treatment during which aromatase inhibitors are given with the primary tumour within the breast. This form of therapy can provide clinical benefits in that patients with large tumours may have these down-staged following successful therapy and the knowledge of (non-) response of the primary tumour may be useful in planning treatment in the adjuvant setting. However, there are also major advantages of using neoadjuvant protocols in the research setting. Because the primary tumour is available for measurement, accurate assessment of response is possible. These measurements may be correlated with putative biological markers in a pre-treatment biopsy of the same tumour. Additionally, since most patients come to definitive surgery, effects of treatment may be monitored by serial samples of individual tumours [8].

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2. Materials and methods

2.1. Patients for neoadjuvant therapy

Postmenopausal patients with large (>3 cm) oestrogen receptor-positive (>20 fmol/mg cytosol protein or Allred score 5–8) primary breast cancers (staged as T_2, T_3, T_{4b}, N_0 or N_1, M_0) have been entered into a series of studies with different endocrine agents. None had received prior treatment with hormonal agents for breast cancer or were taking hormone preparations at the time of study. Tumour size was monitored clinically (by callipers) and by breast ultrasound before and at monthly intervals during treatment. All patients received primary endocrine therapy comprising either letrozole (2.5 or 10 mg daily) or anastrozole (1 or 10 mg daily) or exemestane (25 mg daily) or tamoxifen (20 mg daily).

2.2. Clinical response

Clinical response was based on change in tumour volume taken at monthly intervals over the treatment period. Ultrasound measurement of three orthogonal tumour diameters produced an estimate of tumour volume [9]. Reductions in tumour volume >25% were regarded as evidence of tumour response, those >50% were categorised as major response.

2.3. Pathological response

Sections of the same tumours from pre-treatment and final biopsies were assessed for changes in cellularity and degree of fibrosis. Pathologic response was categorised as: complete when there was no evidence of malignant cells at the original tumour site, microscopic residual when only scattered foci of malignant cells were identified microscopically, partial response when clear decreases in cellularity and/or increases in fibrosis were seen, or no change. The same specimens were evaluated independently for mitotic score [10].

2.4. Immunohistochemistry (IHC)

IHC staining with antibody to Ki67 (MIB1 antigen, DakoCytomation, England) was used as a measure of tumour cell proliferation. Reactivity was detected by an ABC-peroxidase-antiperoxidase (PAP) method and the percentage of cells staining in a minimum of 10 representative high-power microscope fields was used to quantify expression [11]. Reactivity for oestrogen receptor (ER) or progesterone receptor (PgR) was performed by the PAP method, after microwave antigen retrieval, using antibodies clone 6F11 (NovaCastra, England) and clone PGR636 (DakoCytomation, England), respectively, according to the manufacturers' instructions and diaminobenzidine was used as the chromogen. Results were scored on a scale of 0–3 for staining intensity (with each successive score denoting increasing intensity) and on a score of 0–5 for increasing proportion of positive cancer nuclei (0 = none, 1 = <1%, 2 = 1–10%, 3 = 11–33%, 4 = 34–66% and 5 = >66%). The values were then summed into a category score within a range of 0–8 [12]. Scores were independently performed by two observers; divergence scores were resolved by mutual agreement after joint examination of the sections.

Clinical responses were analyzed using Fisher's Exact Test. Differences in tumour histopathological parameters were compared statistically using the Chi-square (χ^2) for trend or Wilcoxon rank tests.

2.5. Oligonucleotide arrays

RNA was extracted from tumour biopsies, amplified and subjected to microarray analysis on Affymetrix chips. Data were analyzed and dendrograms derived. All samples were clustered using hierarchical clustering and Euclidean distances. In order to reduce noise from non-expressed genes, pair-wise differences between samples were derived based on only the present genes (according to Affymetrix' absolute call values). For each individual gene, relative expression was compared in pre-treatment and 10–14 days biopsies in eight patients offered neoadjuvant therapy with letrozole.

3. Results

3.1. Histopathological changes

Letrozole, anastrozole and exemestane were all capable of producing marked changes in tumour morphology. Pathological responses were detected in 66% of tumours treated with the aromatase inhibitors (Table 1). Although in most cases these responses comprised decreased cellularity/increased fibrosis, in a minority of tumours (11%) only microscopic foci of residual disease were evident after 3 months treatment [13]. Complete pathological responses were rarely seen within 3 months treatment, with only one recorded case in a patient treated with letrozole.

Changes in histological grade often accompanied treatment; these usually resulted in a lower score and have been reported elsewhere [13,14]. The grading feature most commonly affected was mitotic score and changes associated with letrozole are summarized in Table 2. Thus, 12 of 23 (52%) tumours showed a marked reduction in mitosis. Interestingly, in a similar group of patients treated over the same time period with tamoxifen, the incidence of decrease was substantially less (17% or 4/24).

Table 1
Pathological responses following neoadjuvant treatment with aromatase inhibitors (letrozole $n = 24$, anastrozole $n = 23$, exemestane $n = 12$)

Complete	1
Minimal residual disease	6
Reduced cellularity	32
No change	20 (34%)

Table 2
Changes in mitotic scores following 3 months treatment with either letrozole or tamoxifen

	Decrease	No change	Increase
Letrozole	12	10	1
Tamoxifen	4	18	2

$p = 0.018$ by Chi-squared test for trend.

Table 3
Changes in progesterone receptor expression following neoadjuvant treatment with aromatase inhibitors (letrozole, anastrozole, exemestane) or tamoxifen

	Decrease	No change	Increase
Aromatase inhibitor	46	3	1
Tamoxifen	12	13	25

$p = 0.008$ by Chi-squared text for trend.

3.2. Immunohistochemistry

The progesterone receptor is regarded as a classical marker of oestrogenic activity, the protein being expressed as a result of oestrogen action mediated by a functional oestrogen receptor. The effect of aromatase inhibitor treatment for 3 months on 50 PgR-positive breast cancers is shown in Table 3 and an illustrative example is shown in Fig. 1. Thus, over 80% of these tumours displayed a marked decrease in staining with therapy and, in about one half of cases, the reduction was to 0. Decreases in staining with treatment were irrespective of the type of inhibitor and pathological response (data not shown). In a small series of PgR-positive cancers treated with tamoxifen, a different pattern of change was seen. Thus, only 17% of cases showed a decrease and the most common change was a paradoxical increase in staining. Changes in pattern of staining were irrespective of clinical or pathological response [13,14]. The changes in pattern of PgR expression with tamoxifen were significantly different from aromatase inhibitor treatment ($\chi^2 = 23.67$; $p < 0.0001$).

The effects of aromatase inhibitors have also been studied on proliferation by using immunohistochemical staining of the marker Ki67. These results are summarized in Table 4 and an illustrative example is shown in Fig. 2. Treatment with AIs was associated with a marked decrease in expression (median decrease 94% in 84% of cases). These effects were irrespective of the type of inhibitor and generally more pronounced than those seen in a matched group of tumours treated with tamoxifen in which, although decreases in proliferation were frequently seen, the incidence and degree of effect was less. In order to determine the timescale of effects on proliferation, a neoadjuvant protocol was developed in which tumour

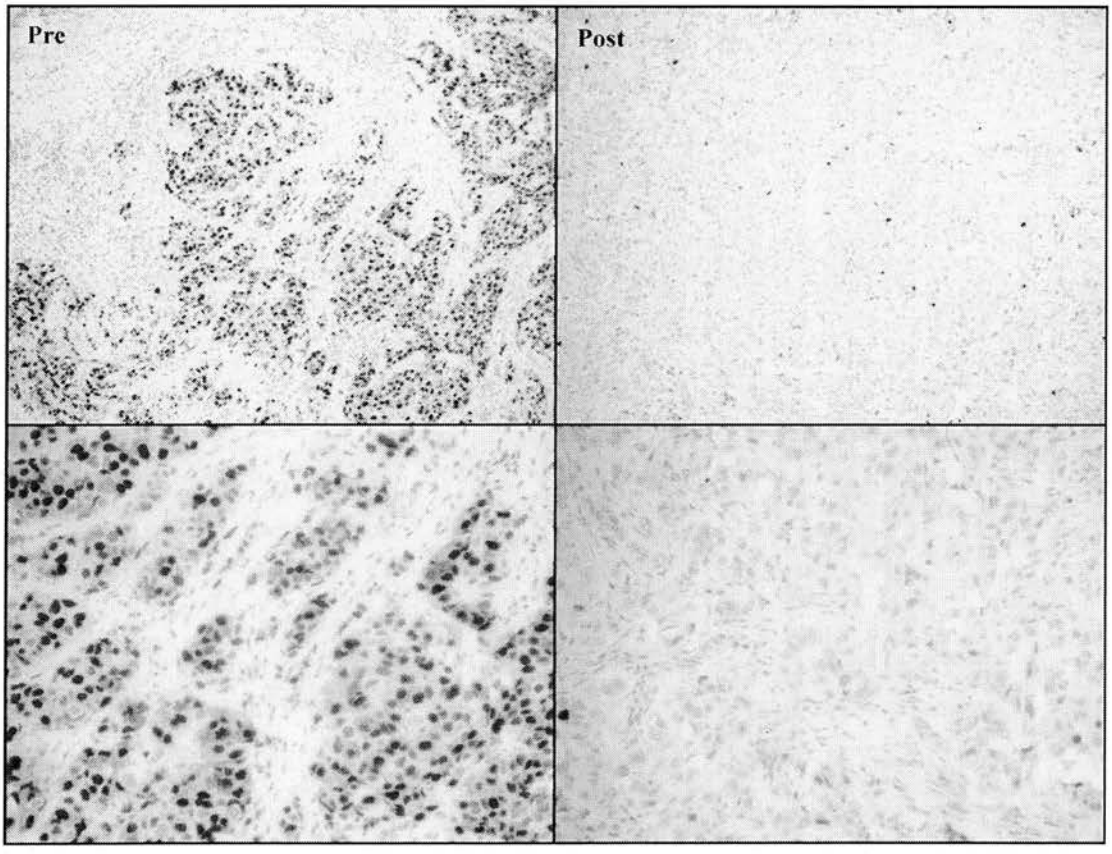

Fig. 1. Progesterone receptor staining of the same tumour before (left) and after (right) treatment with exemestane, showing a major reduction in staining after treatment (original magnification ×200 upper panels, ×500 lower panels).

Table 4
Changes in proliferation as measured by Ki67 staining following neoadjuvant treatment with aromatase inhibitors (letrozole, anastrozole, exemestane) or tamoxifen

	Decrease	
	Cases (%)	Score (%)
Aromatase inhibitor (115)	94	74
Tamoxifen (22)	82	51

The difference between aromatase inhibitors and tamoxifen did not reach statistical significance ($p = 0.07$ by Fisher's Exact Test for % cases and $p = 0.08$ by Wilcoxon rank test for % score).

biopsies were taken before and after 10–14 days and 3 months treatment with letrozole. Of 59 cases, 57 tumours showed a clear decrease in Ki67 staining after 10–14 days of therapy. This decrease was maintained or became even greater by 3 months in 48 cases; in the remaining 9 tumours, the scoring increased (in many cases to levels equivalent or higher than pre-treatment values). In the 2 tumours which did not show a decrease at 10–14 days, there were small decreases at 3 months but the values were still amongst the highest on treatment.

In order to discover novel candidate genes whose expression might be associated or change with response to aromatase inhibitors, tumour biopsies were sequentially taken before and after 10–14 days and 3 months of treatment. These were extracted and the mRNA converted to cDNA and amplified before being subjected to micro-analysis on Affymetrix chips. Microarray analysis of pairs of tumour cores taken before and after 10–14 days of letrozole treatment were compared as shown in Fig. 3 which illustrates the computer generated display for gene changes in 24 tumours. It can be seen that gene changes differ in every single tumour, indicating that there is no single gene signature for response to letrozole. However, there was the suggestion that tumours could be grouped according to amount and incidence of change. Thus, clustering indicated that the cancers could be grouped into 17 cases with greater gene change (left-hand side of the figure) and 7 with lesser changes (right of figure). It remains to determine whether this subdivision relates to clinical/pathological response. Further clustering based on a larger number of tumours (43) is shown in Fig. 4. This suggests that the tumours may be divided into at least four distinct groups of which two may be subdivided further.

4. Discussion

The neoadjuvant setting has proved to be invaluable in terms of elucidating cellular and molecular effects of aromatase inhibitors and evaluating predictive indices of response. Marked changes in tumour morphology and histology may follow the administration of aromatase inhibitors. In ER-rich tumours, effects on histopathological features include changes in cellularity, degree of fibrosis, histological grading features, markers of proliferation and cell death and hormone receptor expression [13,14]. Gross changes in tumour morphology may be seen in the majority of breast cancer by 3 months irrespective of the particular inhibitor. Although these effects are striking, unlike with chemotherapy, these rarely are complete and, at best, microscopic foci of disease remain. Whether with extended treatment more complete remissions would be obtained is still

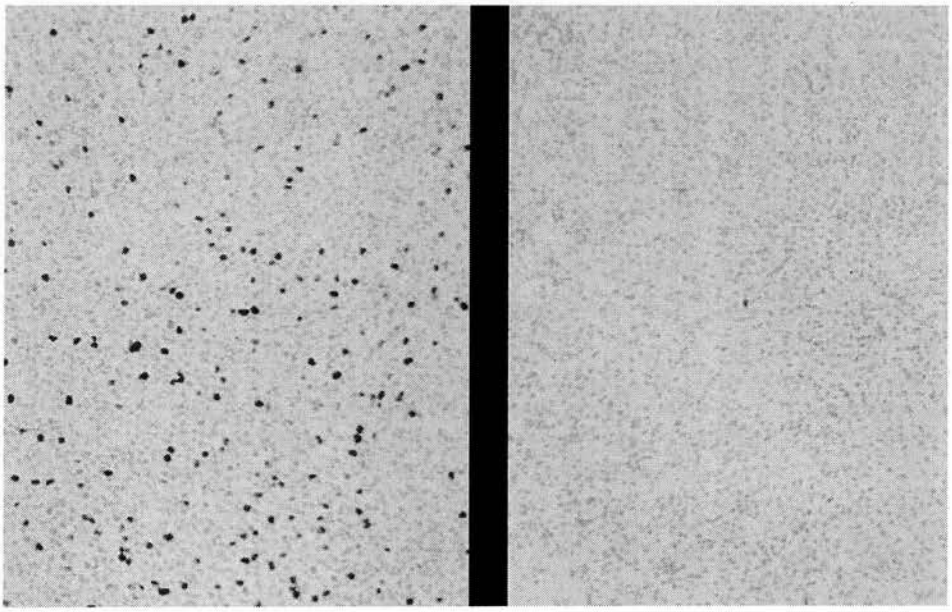

Fig. 2. MIB1 staining of the same tumour before (left) and after (right) 3 months of treatment with letrozole, showing a major reduction in staining after treatment (original magnification ×200).

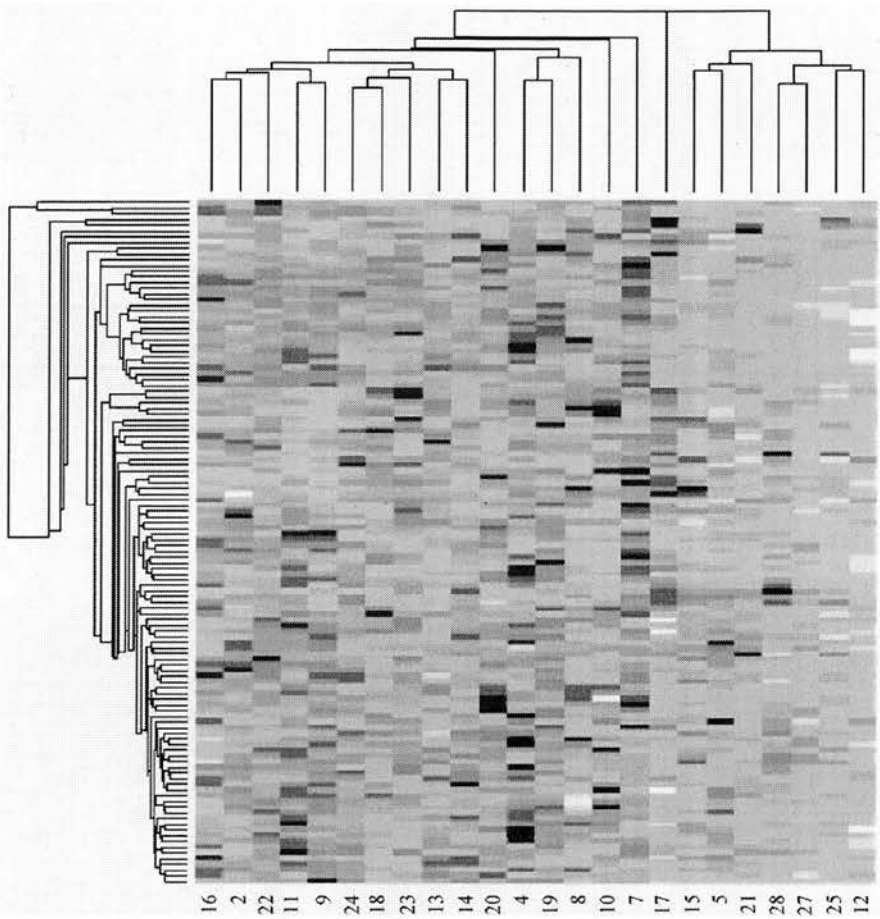

Fig. 3. Microarray analysis of tumour biopsies taken before and after 10–14 days neoadjuvant treatment with letrozole in 24 different patients. Diagrammatic representation of changes in selected cohorts of genes (yellow represents no change in expression and red change in expression (either an increase or a decrease), degree of change is represented by intensity of redness). Tumours are arranged by hierarchical clustering.

a matter of conjecture. Down-staging of histological grade often accompanies gross morphological changes. With aromatase inhibitors, the particular histological feature most often affected was mitotic figures, scores decreasing in the majority of cases. These changes are in keeping with those observed on the proliferation marker, Ki67, which is markedly decreased in 90% of cases after 3 months treatment with an aromatase inhibitor. The higher incidence of effect is probably because mitotic figures are relatively transient events, whereas Ki67 detects cells not only in the process of division but those recently completing the process.

Interestingly, the influence of tamoxifen on mitotic and Ki67 scores are less marked (whereas the effects on other features of tumour histological grade such as tubule formation appear greater) [13]. This probably reflects differences in mechanism of action but differences in the timing or duration of effects cannot be discounted. In this respect, it was of interest to investigate changes occurring with time. To do this, a neoadjuvant protocol was adopted in which sequential biopsies were taken before and after 10–14 days and 3 months of treatment with letrozole. These results show that in almost all ER-rich tumours, marked decreases in proliferation may be seen within days of the onset of treatment. With more extended treatment, proliferation continued to decrease or was maintained in the majority. However, in a subset of tumours, proliferation returned to or exceeded pre-treatment levels. These patterns of change have also been reported for anastrozole [15]. We also have demonstrated that paradoxical lack of change or an increase in proliferation in tumours responding to tamoxifen is associated with early recurrence following primary surgical management [16]. These paradoxical changes in proliferation may therefore represent early evidence of resistance to treatment. Follow-up data subsequent to surgery are not yet available in the cohort of patients treated with letrozole, but clinical and pathology responses at 3 months have been assessed. Tumours failing to show differences in Ki67 staining at either 10–14 days or 3 months still demonstrate substantial reductions in tumour size with treatment. Conversely, reductions in proliferation

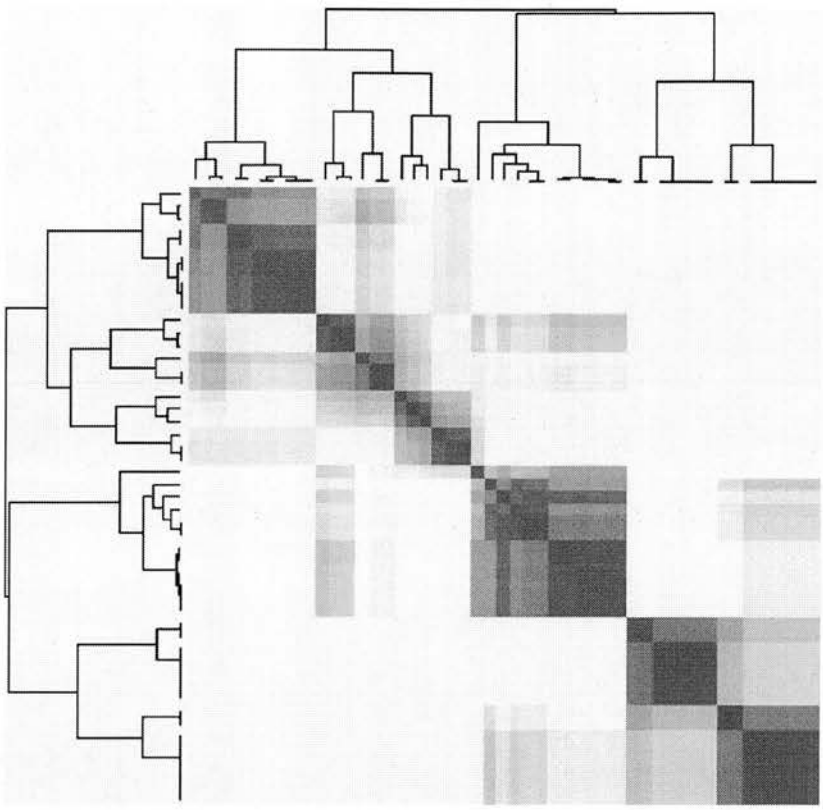

Fig. 4. Hierarchical clustering of changes in gene expression in tumour biopsies taken before and after 10–14 days of neoadjuvant treatment with letrozole in 43 patients. The same dendrograms are plotted vertically and horizontally to produce squares. Colour represents similarity of change in expression with the greatest intensity representing the highest degree of concordance.

did not always translate in clinical response. Clearly, other factors beyond proliferation influence clinical response and change in Ki67 is a poor surrogate for clinical response and other changes in tumour pathology (the reduction in proliferation index in pathological responders to letrozole has been reported to be significantly greater than in those without evidence of response).

Additionally, immunohistochemical staining for progesterone receptors showed that aromatase inhibitors produced marked reduction in the expression of progesterone receptor and, in many cases, staining was not detectable following treatment. These observations would be consistent with oestrogen deprivation and contrast with the effect of tamoxifen which often increased PgR expression [16–20]. Similar effects have been noted on the expression of other oestrogen-regulated markers such as pS2 [21]. Interestingly, loss of PgR expression occurred independently of pathological response [13].

The use of microarray technology has great potential in identifying novel genes which predict for response or are early markers of response but this has yet to make a major impact. However, the technology has not been used to monitor gene changes in substantial cohorts of patients treated with aromatase inhibitors. To this end, we have studied patients treated neoadjuvantly with letrozole and examined expression changes occurring at 10–14 days. To put the effects into perspective, it is worth noting that 22,283 gene sets are expressed on the Affymetrix chip used and that on average 11,456 were expressed in the 59 cases included in the study. Of these, only a single gene was changed by >two-fold in all 59 tumours with treatment. This reflects the observation that every tumour was an individual with regard to a genetic signature change with treatment. This is despite not all tumours responding to treatment. At this time, while molecular clustering analysis is underway, clinical and pathology responses in individual tumours are currently blinded to the investigators. However, as an exercise, it was of interest to look at the pattern of gene changes occurring in at least 50% of tumours (to take account of the expected incidence of response). Interestingly, only 4 genes showed >2-fold change (all decreases) in expression in more than 30 tumours whereas 181 showed a decrease and 334 an increase if the cut-off was relaxed to >1.2-fold. Nevertheless, it was possible to identify by cluster analysis distinct groups of tumours with particular genetic changes with letrozole treatment. It remains to determine whether the data relating to microarray analysis correlate with clinical and pathological response. These analyses are underway. These may permit the early recognition of response/resistance to aromatase inhibitors and help optimize patient management.

5. Summary

In hormone sensitive breast cancer, aromatase inhibitors may produce marked changes in tumour morphology and rapid and dramatic reduction in the expression of markers of proliferation and oestrogen action. Furthermore, microarray analysis may subdivide tumours into distinct groups according to molecular changes.

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References

[1] W.R. Miller, J.M. Dixon, Antiaromatase agents: preclinical data and neoadjuvant therapy, Clin. Breast Cancer 1 (Suppl. 1) (2000) S9–S14.
[2] H.S. Shaw, M.J. Ellis, Letrozole in the treatment of breast cancer, Expert Opin. Pharmacother. 3 (5) (2002) 607–617.
[3] G.M. Keating, B. Jarvis, Letrozole: an updated review of its use in postmenopausal women with advanced breast cancer, Am. J. Cancer 1 (5) (2002) 351–371.
[4] J. Geisler, S. Detre, H. Berntsen, L. Ottestad, B. Lindtjorn, M. Dowsett, P.E. Lonning, Influence of neoadjuvant anastrozole (Arimidex) on intratumoral estrogen levels and proliferation markers in patients with locally advanced breast cancer, Clin. Cancer Res. 7 (5) (2001) 1230–1236.
[5] A.U. Buzdar, J.F. Robertson, W. Eiermann, J.M. Nabholtz, An overview of the pharmacology and pharmacokinetics of the newer generation aromatase inhibitors anastrozole, letrozole, and exemestane, Cancer 95 (9) (2002) 2006–2016.
[6] P.E. Lonning, The role of aromatase inactivators in the treatment of breast cancer, Int. J. Clin. Oncol. 7 (4) (2002) 265–270.
[7] R.W. Brueggemeier, Overview of the pharmacology of the aromatase inactivator exemestane, Breast Cancer Res. Treat. 74 (2) (2002) 177–185.
[8] J.M. Dixon, C.D.B. Love, L. Renshaw, C. Bellamy, D.A. Cameron, W.R. Miller, R.C. Leonard, Lessons from the use of aromatase inhibitors in the neoadjuvant setting, Endocr. Relat. Cancer 6 (1999) 227–230.
[9] W.R. Miller, J.M. Dixon, L. Macfarlane, D. Cameron, T.J. Anderson, Pathological features of breast cancer response following neoadjuvant treatment with either letrozole or tamoxifen, Eur. J. Cancer 39 (2003) 462–468.
[10] C.W. Elston, I.O. Ellis, Pathological prognostic factors in breast cancer I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up, Histopathology 19 (1991) 403–410.
[11] J. Gerdes, M.H. Becker, G. Key, G. Cattoretti, Immunohistological detection of tumour growth fraction (Ki-67 antigen) in formalin-fixed and routinely processed tissues, J. Pathol. 168 (1992) 85–86.
[12] D.C. Allred, J.M. Harvey, M. Berardo, G.M. Clark, Prognostic and predictive factors in breast cancer by immunohistochemial analysis, Mod. Pathol. 11 (1998) 155–168.
[13] W.R. Miller, J.M. Dixon, L. Macfarlane, D. Cameron, T.J. Anderson, Pathological features of breast cancer response following neoadjuvant treatment with either letrozole or tamoxifen, Eur. J. Cancer 39 (2003) 462–468.
[14] T.J. Anderson, J.M. Dixon, M. Stuart, T. Sahmoud, W.R. Miller, Effect of neoadjuvant treatment with anastrozole on tumour histology in postmenopausal women with large operable breast cancer, Br. J. Cancer 87 (2002) 334–338.
[15] M. Dowsett, I. Smith, Greater Ki67 response after 2 weeks neoadjuvant treatment with anastrozole (A) than with tamoxifen (T) or anastrozole plus tamoxifen (C) in the IMPACT trial: a potential predictor of relapse-free survival, Breast Cancer Res. Treat. 82 (Suppl. 1) (2003) 6.
[16] W.R. Miller, T.J. Anderson, R.A. Hawkins, J. Keen, J.M. Dixon, Neoadjuvant endocrine treatment: the Edinburgh experience, in: A. Howell, M. Dowsett (Eds.), Primary Medical Therapy for Breast Cancer. ESO Scientific Updates, vol. 4, Elsevier Science BV, Amsterdam, 1999, pp. 1–11.
[17] J.C. Keen, J.M. Dixon, E.P. Miller, D.A. Cameron, U. Chetty, A. Hanby, C. Bellamy, W.R. Miller, The expression of Ki-S1 and BCL-2 and the response to primary tamoxifen therapy in elderly patients with breast cancer, Breast Cancer Res. Treat. 44 (1997) 123–133.
[18] A. Makris, T.J. Powles, D.C. Allred, M. Dowsett, Changes in hormone receptors and proliferation markers in tamoxifen-treated breast cancer patients and the relationship with response, Breast Cancer Res. Treat. 48 (1998) 11–20.
[19] J. Chang, T.J. Powles, D.C. Allred, S.E. Ashley, A. Makris, R.K. Gregory, C.K. Osborne, M. Dowsett, Prediction of clinical outcome from primary tamoxifen by expression of biologic markets in breast cancer patients, Clin. Cancer Res. 6 (2000) 616–621.
[20] R.D. Johnstone, I.E. Smith, M. Dowsett, Place of aromatase inhibitors in the endocrine therapy of breast cancer, in: W.R. Miller, R.J. Santen (Eds.), Aromatase Inhibition and Breast Cancer, Marcel Dekker, New York, 2001, pp. 29–50.
[21] M.J. Ellis, A. Coop, B. Singh, L. Mauriac, A. Llombert-Cussac, F. Janicke, W.R. Miller, D.B. Evans, M. Dugan, C. Brady, E. Quebe-Fehling, M. Borgs, Letrozole is more effective neoadjuvant endocrine therapy than tamoxifen for ErbB-1 and/or ErbB-2 positive, estrogen receptor-positive primary breast cancer: evidence from a phase II randomized trial, J. Clin. Oncol. 19 (2001) 3808–3816.

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Surgical issues surrounding use of aromatase inhibitors☆

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Abstract

There are important surgical issues related to the use of the third generation aromatase inhibitors in both the neoadjuvant and adjuvant settings. Neoadjuvant hormone therapy is effective at downstaging tumours, particularly large tumours initially thought to be inoperable or requiring mastectomy. Randomised trials have shown that the newer aromatase inhibitors letrozole and anastrozole increase the numbers of women who are suitable for breast-conservation compared with tamoxifen, and that letrozole is superior to tamoxifen in terms of clinical response.

Aromatase inhibitors are most effective in ER-rich tumours and are clinically and biologically effective in both HER2 positive and negative tumours, whereas HER2 positive tumours show a level of resistance to tamoxifen.

In neoadjuvant studies comparing aromatase inhibitors with tamoxifen, the duration of use has been 3–4 months, by which time any response is usually evident but longer treatment periods produce continued shrinkage and response. The re-excision rate following breast conservation surgery after neoadjuvant hormone therapy is favourable compared with the rates following immediate wide local excision. Local recurrence rates are acceptable in patients undergoing neoadjuvant therapy and breast-conserving surgery providing post-operative radiotherapy is given.

Adjuvant aromatase inhibitors, as well as having an effect on metastatic disease and survival, reduce local and regional recurrence.

Keywords: Aromatase inhibitors; Neoadjuvant; Adjuvant; Breast cancer; Surgical outcomes

1. Introduction

There are important surgical issues related to the use of the new generation of aromatase inhibitors in both the neoadjuvant and adjuvant settings. Large operable and locally advanced breast cancers continue to be common despite the introduction of breast screening programmes [1]. Neoadjuvant (pre-operative or primary) systemic chemotherapy often results in locally advanced and unresectable primary breast tumours becoming operable [2,3]. Neoadjuvant hormone therapy has also been used more recently to shrink large operable breast cancers that would normally require mastectomy allowing them subsequently to be treated with breast-conserving surgery [4–14]. When breast-conserving surgery is feasible for a patient, neoadjuvant therapy may allow a less extensive resection and so improve the final cosmetic outcome.

Selecting which patients will benefit and the optimal agent in this setting has been addressed in a number of studies comparing tamoxifen and aromatase inhibitors. What is not clear is how long patients should receive neoadjuvant hormone therapy before operation. Most studies have treated patients for 3–4 months and have reported high rates of conversion from mastectomy to breast conserving surgery. There remains as yet little data on the rates of complete excision and local recurrence after down staging with neoadjuvant endocrine therapy.

Randomised trials of aromatase inhibitors in both the adjuvant and extended adjuvant settings have shown that these agents reduce local recurrence after either mastectomy or breast conserving surgery and reduce the rate of new breast primary cancers in both the treated and contra-lateral breast [15,16]. This is important both for the patient and sur-

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geon because it reduces the need for surgical intervention in patients who are usually otherwise well and have no clinical evidence of systemic metastases.

2. Selection for treatment

Patients selected for neoadjuvant treatment need to potentially gain benefit from therapy. Eligible patient groups are outlined in Table 1. The greatest experience with neoadjuvant endocrine therapy has been in postmenopausal women initially with tamoxifen but more recently with the third generation aromatase inhibitors.

3. Selecting the optimal agent to use in neoadjuvant endocrine setting

Four randomized trials comparing tamoxifen and the newer aromatase inhibitors have been performed. The first randomised trial (P024) compared 4 months of neoadjuvant treatment with either letrozole or tamoxifen in 324 postmenopausal women with ER+ and/or PgR+ breast cancer. Evaluation of all patients indicated that letrozole achieved a significantly higher clinical response rate than tamoxifen (55% versus 36%; $P<0.001$), enabling significantly more letrozole-treated patients than tamoxifen-treated patients to undergo breast-conserving surgery (45% versus 35%; $P=0.022$) (Fig. 1) [6]. In this study, even in patients with locally advanced breast cancer significantly more patients treated by letrozole were eligible for breast conserving surgery (Fig. 2).

The second neoadjuvant study, designated IMPACT (immediate pre-operative arimidex compared to tamoxifen) compared anastrozole (1 mg daily) versus tamoxifen (20 mg daily) versus anastrozole plus tamoxifen [7]. This was a multicentre, randomised, double-blind trial, with 330 patients. Patients were postmenopausal, with ER+ and/or PgR+ breast cancer that was large and operable (T2 or T3, N0–2, M0)

Table 1
Selection of patients for Neoadjuvant therapy based on likely benefit

Patients who will benefit
Locally advanced breast cancer who may become operable
Large operable tumours
Converting from mastectomy to breast-conserving surgery
Improving cosmetic outcome of patients with larger cancers suitable for conserving surgery
Patients who are unlikely to benefit
Those who have operable disease but have multiple tumours who will require mastectomy regardless of pre-operative treatment success
Those who have tumours that respond very slowly or indiscernibly to treatment, such as many of the invasive lobular carcinomas
Those with tumours where a reduction in tumour volume is not achievable within a reasonable time period, such as invasive mucinous carcinomas

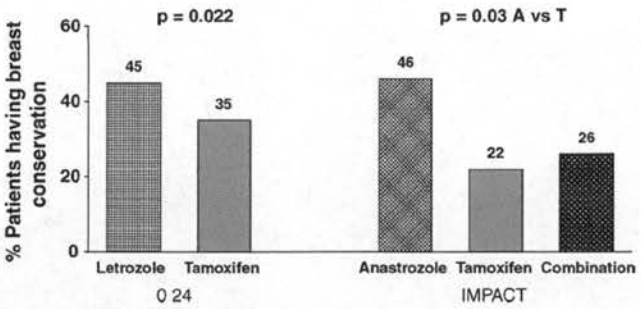

Fig. 1. Rates of conversion to breast conservation in P024 study comparing 4 months of neoadjuvant letrozole and tamoxifen and IMPACT study comparing 3 months of neoadjuvant anastrozole alone, tamoxifen alone, or the two agents combined.

or potentially operable, locally advanced (T4b, N0–2, M0). Treatment was for 3 months prior to surgery. Results presented at San Antonio in 2003 demonstrated that although there was no difference in response rates between groups (using both ultrasound and caliper measurements, there was a higher rate of conversion of patients from mastectomy to breast-conserving surgery with anastrozole. Out of the 124 patients deemed by the surgeon to be suitable for a mastectomy at baseline, 45.7%, 22.2% and 26.2% in the Anastrozole, Tamoxifen and Combined groups, respectively, became eligible for breast-conserving surgery (Fig. 1).

A second European study comparing 3 months of neoadjuvant anastrozole and tamoxifen designated PROACT (preoperative anastrozole compared with tamoxifen) recruited 451 patients [8]. Approximately 30% of patients received concomitant neoadjuvant chemotherapy. This study failed to show any statistical difference in response between the two hormonal agents in the whole group and in patients who received hormonal agents alone. Patients who were inoperable or needed a mastectomy at baseline were more likely either to become operable or have breast conserving surgery if they were treated with anastrozole $P=0.003$.

A small study from Russia involving only 73 patients compared 3 months of neoadjuvant tamoxifen or exemestane. The overall response rate and the rate of breast conserving surgery were significantly greater (both $P<0.05$) in patients treated by exemestane [9].

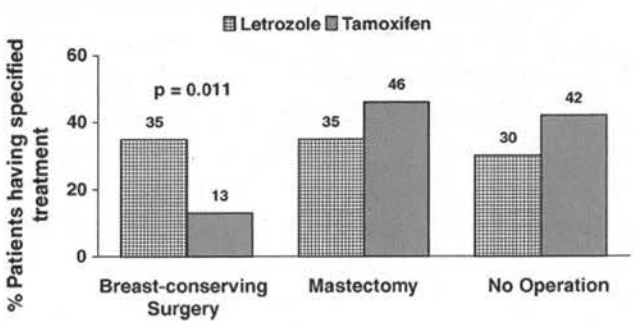

Fig. 2. Outcomes of 83 patients with inoperable (locally advanced) breast cancer in the P024 study which compared 4 months of neoadjuvant tamoxifen and letrozole.

Table 2
Responses in trial P024 comparing 4 months of neoadjuvant letrozole vs. tamoxifen, relative to confirmed ER and/or PgR status [10]

Agent	Marker status	Response rate (%)	*P*-value
Letrozole	ER+	60	0.005
	ER−	19	
	PgR+	63	0.018
	PgR−	41	
Tamoxifen	ER+	40	0.031
	ER−	11	
	PgR+	43	0.076
	PgR−	28	

ER, oestrogen receptor; PgR, progesterone receptor.

Aromatase inhibitors are now the neoadjuvant hormonal agents of choice in appropriately selected postmenopausal women with hormone sensitive breast cancers. The data are most impressive for letrozole. Apart from benefiting patients, the neoadjuvant setting provides an opportunity to sample tumours during treatment and correlate biological changes with response. Not all treated patients do respond, so selection for treatment is thus critical.

4. Predicting response to neoadjuvant endocrine therapy

In early studies with tamoxifen, patients were not selected for treatment on the basis of oestrogen receptor-positive (ER+) or progesterone receptor-positive (PgR+) status, which identifies those patients most likely to respond [10]. Nevertheless, one early study concluded that tamoxifen provided an alternative pre-operative treatment option for operable breast cancer in elderly patients [11]. Subsequent studies in postmenopausal ER+ patients demonstrate substantial tumour volume reductions over a 3–4 month treatment period using a variety of endocrine agents, including tamoxifen and the third-generation aromatase inhibitors, letrozole, anastrozole, and exemestane [6,10,12–14].

In the P024 study, tumour responses to letrozole versus tamoxifen were also evaluated according to biopsy-confirmed ER and/or PgR status [10]. Both letrozole and tamoxifen achieved significantly more responses in patients with ER+ tumours than those with ER-negative tumours (Table 2).

In both ER+ and ER-cancers (these cancers were PgR positive), response rates were higher for letrozole, reflecting the significantly better overall response rate for letrozole versus tamoxifen in biopsy-confirmed patients (60% versus 41%; $P = 0.004$). Responses to letrozole were significantly better for PgR+ tumours than for PgR-negative tumours, and a similar but weaker trend was also seen with tamoxifen. Differences in response rates between these two agents were most marked for tumours that were both ER+ and/or PgR+ and also positive for the markers ErbB-1 and/or ErbB-2 (response rate to letrozole 88% versus 21% for tamoxifen; $P = 0.0004$) [10].

There was also some evidence of a direct correlation between the degree of ER expression and the incidence and extent of tumour response [17,18]. This is true for both postmenopausal and premenopausal women treated by neoadjuvant endocrine therapy. ER-rich tumours can be characterised by several criteria. In the P024 randomised trial of pre-operative letrozole versus tamoxifen, clinical responses were related to the level of ER expression as determined by immunohistochemistry (IHC) using the semi-quantitative Allred scoring system (0–8). There were no tamoxifen-induced responses at ER levels below a score of 6, in contrast to letrozole-induced responses of >30% at a score of 3 (Fig. 3).

5. Response and HER2 status

As previously mentioned, the P024 study showed that there appeared to be a particular difference in response rates in tumours that were ER and/or PgR positive and also over expressed erbB1 and/or erbB2 (HER1 or HER2)[10]. Furthermore, it was noticed that there was a significantly lower response to tamoxifen in erbB1 and erbB2 positive tumours than in erbB1 and erbB2 negative cancers (14% versus 41%; $P = 0.01$). In contrast, it was found that there was a less dramatic difference in the effect of letrozole on erbB1 and erbB2 positive or negative tumours (88% versus 54%).

Similar results have been shown in neoadjuvant use of anastrozole. The IMPACT study found that for those patients whose tumours had HER2 overexpression (34 of

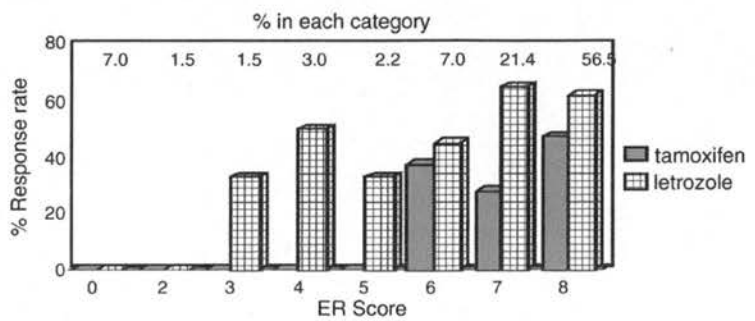

Fig. 3. Clinical response rate vs. ER Allred score for letrozole and tamoxifen in the P024 randomised trial. Reprinted with permission from Ellis et al. [10].

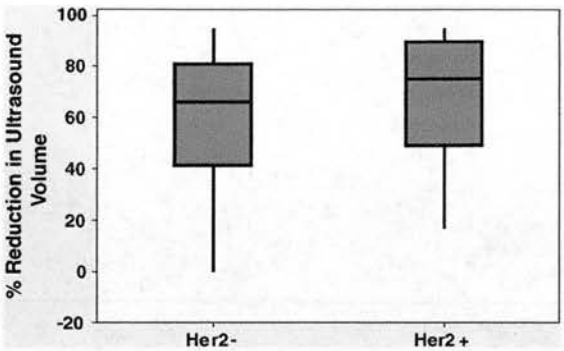

Fig. 4. Clinical response to neoadjuvant anastrozole in HER2 positive ($n=6$) and negative ($n=16$) tumours [20].

239 tumours), anastrozole had a higher clinical response rate (58%) than tamoxifen (22%; $P=0.09$) [7].

The relationship of response to aromatase inhibitors and ErbB2 status has also been investigated in two Edinburgh studies. One hundred and seventy-two postmenopausal women with large operable or locally advanced ER-rich (ER Allred score >6) breast cancers have been treated with 3 months of neoadjuvant letrozole [19]. ErbB2 status was assessed by Hercept test and FISH for >2 samples, and response assessed by clinical and ultrasound examination. Of 172 patients, 18 had tumours that were classified as ErbB2 positive. At 3-month assessment there was no significant difference in clinical response between ErbB2 positive and negative tumours (61% versus 69%; $P=0.506$), confirming the equal efficacy of letrozole in both ErbB2 positive and negative cancers.

In a second series 24 postmenopausal women with oestrogen receptor-rich, large, operable breast tumours received 3 months' neoadjuvant anastrozole [13]. Twenty-two patients had sufficient tumour in all their specimens to allow staining for erbB2 status prior to treatment and also to study changes in proliferation as assessed by Ki67 antibody and PgR as assessed by the DAKO antibody [20]. There were 6 erbB2 >3 tumours with the other 16 tumours being either negative or >1. There was no difference in clinical response between the two groups. Changes in proliferation and PgR receptor did not differ between the different groups, demonstrating that anastrozole is equally effective both clinically and biologically erbB2 positive and negative tumours (Fig. 4).

6. Duration of neoadjuvant endocrine therapy

The optimal duration of neoadjuvant endocrine therapy has yet to be established. In early studies, patients usually remained on tamoxifen until their tumours became unresponsive and grew [11,21]. In unpublished studies from the Edinburgh Breast Unit, 3 months has been identified as the most appropriate initial length of pre-operative treatment. This was determined in a consecutive series of 100 patients who were more than 70 years old and who had ER-rich breast cancers (>20 fmol/mg of cytosolic protein). After 3 months of tamoxifen, 72 patients had responded, based on a >25% ultrasound tumour volume reduction, and 1 patient had progressing disease. The remaining 27 patients were continued on tamoxifen for an additional 3 months, during which 4 responded while 5 progressed. From these data, it is evident that if a patient has not responded within 3 months, then the subsequent poor response-to-progression ratio does not warrant more prolonged treatment. By 3 months the numbers developing disease progression seems to offset any benefit to the few whose tumours respond after initially being static Therefore, in Edinburgh, after 3 months of pre-operative endocrine treatment response is formally assessed and patients who have not responded are advised that continuation of the same treatment is unlikely to be effective and an alternative treatment is necessary. Responders are advised either to have surgery or to continue on hormone therapy.

Most neoadjuvant studies with aromatase inhibitors have treated patients for 3 or 4 months, and by this time many patients' tumours have responded sufficiently to downstage the surgical procedure required to excise the cancer. Some, however, remain inoperable or still require mastectomy. A prospective audit of 142 postmenopausal women with large operable or locally advanced ER-rich (ER Allred score >6) breast cancer has been carried out in Edinburgh assessing clinical response to letrozole [22]. After 3 months 100 patients had responded, this included some patients whose disease was inoperable and were now suitable for operation either by mastectomy or breast conserving surgery, and others who had large operable cancers that had reduced in size sufficiently to allow breast-conserving surgery. Forty-two patients had either disease that remained inoperable, had large cancers not yet suitable for breast conserving surgery or refused or were considered unfit for surgery because of other comorbidities and continued on letrozole for at least 3 further months. Twenty-two were still taking letrozole at 12 months. The median % reduction in clinically measured tumour volumes in these women was 52% from 0 to 3 months. There was a further 57% volume reduction from baseline at 3 months between 3 and 6 months and another 66% reduction from baseline at 6 months between 6 and 12 months, showing that tumours continued to reduce in volume during the 12-month study period. The complete response rate also increased over time, with 9.5% at 3 months, 29% at 6 months and 36% at 12 months. Only one patient who had an initial response at 3 months had disease progression in this cohort. These data suggest that letrozole can be used safely for up to 12 months. The optimum duration of neoadjuvant endocrine therapy has yet to be defined and further studies are indicated.

7. Completeness of tumour excision and incidence of local recurrence following neoadjuvant therapy

In study of neoadjuvant chemotherapy, the histology of wide excision specimens following downstaging revealed

Table 3
Outcomes for patients in Edinburgh having undergone initial wide local excision

	Total WLE	Re-excision *n* (%)	Mastectomy *n* (%)	Single BCS procedure (%)
No pre-operative treatment	1374	156 (11.4%)[a]	78 (5.7%)	1140 (83%)[b]
Neoadjuvant AI	147	6 (4.1%)[a]	6 (4.1%)	135 (92%)[b]

WLE, wide local excision; BCS, breast conserving surgery.
[a] $P=0.007$.
[b] $P=0.006$.

that in 16% of 227 cases there was evidence of multifocality of tumour, with the frequency greatest for larger tumours [23]. A series of 25 patients in whom breast-conservation surgery was performed after chemotherapy in Edinburgh revealed similar results: 6 patients had diffuse or multifocal disease on pathologic examination, even though there was no palpable tumour in 5 of these.

Data from Edinburgh of all wide local excisions performed in patients who either did or did not have pre-operative treatment have been analysed, and have shown favourable results for neoadjuvant endocrine therapy. Of 1374 patients who underwent wide local excision without any pre-operative treatment, 156 (11.4%) required re-excision of margins, and 78 (5.7%) required mastectomy. In a series of 147 patients treated during the same time period who underwent breast conserving surgery after neoadjuvant therapy significantly less ($n=6$ (4.1%); $P=0.007$) of these women required a re-excision; 6 patients (4.1%) in this group did require mastectomy. There were a significantly greater proportion of patients in the neoadjuvant group who had successful breast conservation at a single procedure than in those who had no pre-operative treatment (92% versus 83%; $P=0.006$) (Table 3).

Several studies have examined rates of tumour recurrence following neoadjuvant therapy and surgery (and in some cases local radiation therapy). Veronesi et al. reported 12 cases of local recurrence in 203 patients (6% incidence) at a mean follow-up of 3 years after pre-operative chemotherapy followed by quadrantectomy and local radiotherapy. This was considerably better than the 22% recurrence rate among comparable patients who underwent mastectomy [23]. In another trial, recurrence rates were similar for patients receiving chemoendocrine therapy pre-operatively and for those receiving only adjuvant treatment (3.5% and 2.7%, respectively, at 48 months' median follow-up) [24]. The importance of surgically removing tumours following complete responses to treatment is emphasised by a report that the rate of recurrence was significantly higher for *complete* responders who then received radiotherapy without surgery, as compared to *partial* responders who had surgery [25].

As yet there are few reported data on local recurrence after breast-conserving surgery in large numbers of patients following neoadjuvant endocrine therapy. Fig. 5 shows local recurrences in the series of patients treated in Edinburgh, with a distinction made between use of surgery alone versus surgery plus radiotherapy following pre-operative treatment.

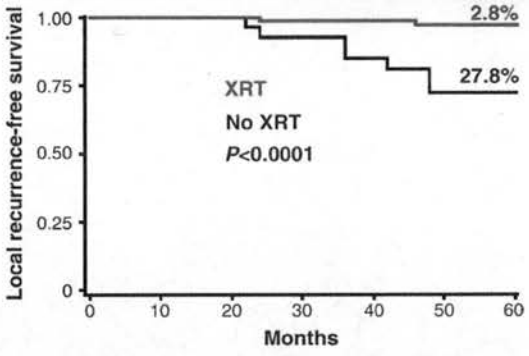

Fig. 5. Effect of radiotherapy on local recurrence in patients treated with neoadjuvant endocrine therapy followed by breast-conserving surgery.

At 5 years there was only a 2.8% local recurrence rate in patients treated with neoadjuvant endocrine therapy and then breast conserving surgery followed by radiotherapy, compared with a 27.8% rate of local recurrence in those treated by breast conserving surgery without radiotherapy. It is now our policy to give radiotherapy after operation to all women deemed fit enough.

8. Adjuvant aromatase inhibitors

Two large trials have presented data on the effects of adjuvant or extended adjuvant therapy on local and regional recurrences. These have important implications for surgeons. In the ATAC trial, 9366 patients who had completed primary therapy and were eligible for adjuvant endocrine therapy were treated with either anastrozole or tamoxifen alone, or a combination of both [15]. Results at 4 years revealed that anastrozole had a significantly longer disease free survival period than tamoxifen, and in hormone receptor tumours there was a significant reduction in development of new primary breast cancer with anastrozole compared with tamoxifen (Fig. 6).

Similar results have been found with letrozole. The MA17 trial randomised 5187 postmenopausal women who had finished primary treatment for operable breast cancer and 5 years adjuvant tamoxifen to receive 5 years letrozole or placebo [16]. At interim analysis there were fewer locoregional recurrences and fewer new primary tumours in the contralateral breast in the letrozole group than in the placebo group.

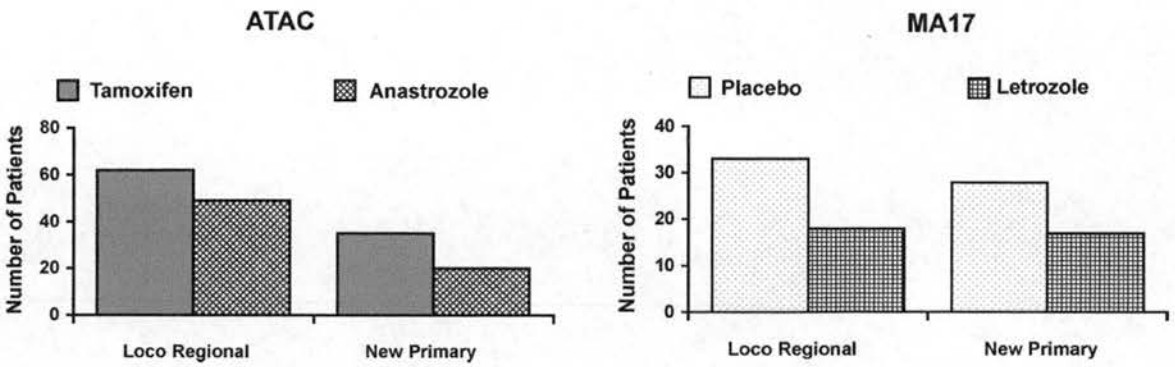

Fig. 6. Results of adjuvant studies ATAC and MA17: locoregional recurrence and incidence of new primary breast tumour.

9. Conclusions

Neoadjuvant hormone therapy is effective at downstaging tumours, particularly large tumours initially thought to be inoperable or requiring mastectomy. Randomised trials have shown that the newer aromatase inhibitors letrozole and anastrozole increase the numbers of women who are suitable for breast-conservation compared with tamoxifen, and that letrozole is superior to tamoxifen in terms of clinical response.

Careful selection of those likely to benefit from neoadjuvant therapy is required. Aromatase inhibitors are most effective in ER-rich tumours, although letrozole is effective at even low ER Allred scores ($\leq$5), whereas tamoxifen is not. The aromatase inhibitors are clinically and biologically effective in both HER2 positive and negative tumours, whereas HER2 positive tumours show a level of resistance to tamoxifen.

In neoadjuvant studies comparing aromatase inhibitors with tamoxifen, the duration of use has been 3–4 months, by which time any response is usually evident. In patients who show a response to letrozole but were unfit for surgery or continued because their disease remained inoperable at 3–4 months, letrozole used for up to 12 months is associated with continued response.

Reports to date suggest that the re-excision rate in neoadjuvant hormone therapy groups is favourable compared with groups undergoing standard treatment with wide local excision, with significantly more neoadjuvant patients having successful breast conservation with a single procedure. Local recurrence rates are acceptable in patients undergoing neoadjuvant therapy and breast-conserving surgery if this is combined with post-operative radiotherapy.

Adjuvant aromatase inhibitors, as well as having an effect on metastatic disease and survival, reduce local and regional recurrence, which is important both for the patient and the surgeon.

References

[1] H.D. Bear, Indications for neoadjuvant chemotherapy for breast cancer, Semin. Oncol. 25 (2 Suppl. 3) (1998) 3–12.
[2] M. Perloff, G.J. Lesnick, Chemotherapy before and after mastectomy in stage III breast cancer, Arch. Surg. 117 (7) (1982) 879–881.
[3] P. Schick, J. Goodstein, J. Moor, J. Butler, K.L. Senter, Preoperative chemotherapy followed by mastectomy for locally advanced breast cancer, J. Surg. Oncol. 22 (4) (1983) 278–282.
[4] J.M. Dixon, T.J. Anderson, W.R. Miller, Neoadjuvant endocrine therapy of breast cancer: a surgical perspective, Eur. J. Cancer 38 (17) (2002) 2214–2221.
[5] J.M. Dixon, Neoadjuvant therapy in postmenopausal women, in: J.N. Ingle, M. Dowsett (Eds.), Advances in Endocrine Therapy of Breast Cancer: Procedures of the 2003 Gleneagles Conference, Summit Communications, New York, 2004, pp. 73–85.
[6] W. Eiermann, S. Paepke, J. Appfelstaedt, et al., Preoperative treatment of postmenopausal breast cancer patients with letrozole: a randomized double-blind multicenter study, Ann. Oncol. 12 (11) (2001) 1527–1532.
[7] I. Smith, M. Dowsett, on behalf of the IMPACT Trialists, Comparison of anastrozole vs. tamoxifen alone and in combination as neoadjuvant treatment of estrogen receptor-positive (ER+) operable breast cancer in postmenopausal women: the IMPACT trial, Breast Cancer Res. Treat. 82 (supp 1) (2003) S6.
[8] L. Cataliotti, on behalf of the PROACT trialists, Efficacy of Preoperative Anastrozole Compared with Tamoxifen in postmenopausal women with hormone receptor positive breast cancer, Presented at 4th European Breast Cancer Conference Hamburg, Germany, 17 March 2004.
[9] V.F. Semiglazov, V.V. Semiglazov, V.G. Ivanov, et al., Neoadjuvant endocrine therapy: exemestane (E) versus tamoxifen (T) in postmenopausal ER+ breast cancer patients (T1-4, N1-2, M0), Breast Cancer Res. Treat. 82 (supp 1) (2003) S22.
[10] M.J. Ellis, A. Coop, B. Singh, et al., Letrozole is more effective neoadjuvant endocrine therapy than tamoxifen for ErbB-1- and/or ErbB-2-positive, estrogen receptor-positive primary breast cancer: evidence from a phase III randomized trial, J. Clin. Oncol. 19 (18) (2001) 3808–3816.
[11] J.M. Horobin, P.E. Preece, J.A. Dewar, R.A.B. Wood, A. Cushieri, Long-term follow-up of elderly patients with locoregional breast cancer treated with tamoxifen only, Br. J. Surg. 78 (2) (1991) 213–217.
[12] J.M. Dixon, C.D. Love, C.O. Bellamy, et al., Letrozole as primary medical therapy for locally advanced and large operable breast cancer, Breast Cancer Res. Treat. 66 (3) (2001) 191–199.
[13] J.M. Dixon, L. Renshaw, C. Bellamy, M. Stuart, G. Hoctin-Boes, W.R. Miller, The effects of neoadjuvant anastrozole (Arimidex) on tumor volume in postmenopausal women with breast cancer: a randomized, double-blind, single-center study, Clin. Cancer Res. 6 (6) (2000) 2229–2235.
[14] J.M. Dixon, T. Anderson, W.R. Miller, Phase IIb study of neoadjuvant exemestane (EXE) in locally advanced breast cancer, Proc. Am. Soc. Clin. Oncol. 20 (2001) 40b (Abstract 1908).
[15] ATAC Trialists' Group, Anastrozole alone or in combination with tamoxifen versus tamoxifen alone for adjuvant treatment of post-

menopausal women with early breast cancer: first results of the ATAC randomised trial, Lancet 359 (2002) 2131–2139.
[16] P.E. Goss, J.N. Ingle, S. Martino, et al., A Randomized trial of letrozole in postmenopausal women after five years of tamoxifen therapy for early-stage breast cancer, N. Engl. J. Med. 349 (2003) 1793–1802.
[17] J.C. Keen, J.M. Dixon, E.P. Miller, et al., The expression of KiS1 and BCL-2 and the response to primary tamoxifen therapy in elderly patients with breast cancer, Breast Cancer Res. Treat. 44 (2) (1997) 123–133.
[18] W.R. Miller, T.J. Anderson, R.A. Hawkins, J. Keen, J.M. Dixon, Neoadjuvant endocrine treatment: the Edinburgh experience, in: A. Howell, M. Dowsett (Eds.), Primary Medical Therapy for Breast Cancer: Clinical and Biological Aspects, vol. 4, Elsevier Science B.V., Amsterdam, 1999, pp. 89–99.
[19] O. Young, J. Murray, L. Renshaw, et al., Neoadjuvant letrozole is equally effective in HER2 positive and negative breast cancers, Breast Cancer Res. Treat. 88 (supp 1) (2004) S38.
[20] J.M. Dixon, J. Jackson, M. Hills, et al., Anastrozole demonstrates clinical and biological effectiveness in erbB2 ER positive breast cancers, Eur. J. Cancer 40 (18) (2004) 2742–2747.
[21] J. Bradbeer, J. Kyngdon, Primary treatment of breast cancer in elderly women with tamoxifen, Clin. Oncol. 9 (1) (1983) 31–34.
[22] L. Renshaw, J. Murray, D. Cameron, et al., Is there an optimal duration of neoadjuvant therapy? Breast Cancer Res. Treat. 88 (supp 1) (2004) S36.
[23] U. Veronesi, G. Bonadonna, S. Zurrida, et al., Conservation surgery after primary chemotherapy in large carcinomas of the breast, Ann. Surg. 222 (5) (1995) 612–618.
[24] A. Makris, T.J. Powles, S.E. Ashley, et al., A reduction in the requirements for mastectomy in a randomised trial of neoadjuvant chemoendocrine therapy in primary breast cancer, Ann. Oncol. 9 (11) (1998) 1179–1184.
[25] P. Ellis, S. Ashley, G. Walsh, et al., Identification of clinical factors predicting outcome following primary chemotherapy for operable breast cancer, Breast 8 (1999) 235.

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Neoadjuvant tamoxifen and aromatase inhibitors: comparisons and clinical outcomes☆

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Abstract

Neoadjuvant hormonal therapy for oestrogen receptor (ER) and/or progesterone receptor (PgR) positive large operable or locally advanced breast cancer is effective and a safe alternative to chemotherapy in postmenopausal women. A randomised trial has demonstrated that the response rate and the incidence and degree of downstaging with the aromatase inhibitor letrozole is significantly greater than with tamoxifen [J. Clin. Oncol. 19 (2001) 3808]. Tumours at all levels of ER appear to respond better to letrozole than tamoxifen but at low levels of ER responses are seen only with letrozole and not with tamoxifen. Patients most likely to benefit from neoadjuvant therapy and those who achieve the greatest reduction in tumour volume are those patients with tumours that express very high levels of ER (ALLRED category score 8). Both letrozole and anastrozole appear effective in both erbB2 positive and negative breast cancers. Three months of treatment is adequate to determine if a tumour will respond. Following breast-conserving surgery and radiotherapy, local recurrence rates appear satisfactory.

Keywords: Neoadjuvant tamoxifen; Aromatase inhibitors; ER and/or PgR positive

1. Introduction

Until recently neoadjuvant therapy of breast cancer has been used predominantly as cytotoxic chemotherapy [1–4]. Endocrine treatment is now emerging as an attractive alternative in hormone receptor positive postmenopausal women many of whom who could not tolerate the toxicities of chemotherapy. There have been few controlled studies of neoadjuvant endocrine therapy. In early studies, tamoxifen was used but patients were not selected on the basis of having oestrogen receptor (ER) or progesterone receptor (PgR) positive breast cancers to identify those most likely to respond [5].

1.1. Studies with tamoxifen

Randomised trials comparing primary endocrine therapy with tamoxifen alone with surgery±tamoxifen have all been in elderly patients [6–9]. Patients in these studies were not routinely selected on the basis of having ER or PgR positive breast cancer. In two of the studies, tamoxifen was compared with immediate surgery alone and in the other two, tamoxifen was compared with surgery and tamoxifen [6,7]. The time to relapse or first event was significantly shorter in the tamoxifen alone arm, as would be expected. A more recent combined analysis of these trials [10] showed that this translated to a significant reduction in breast cancer deaths in the immediate surgery group. However, none of these trials were designed to see whether there was a difference in survival between patients treated by neoadjuvant endocrine therapy before surgery or surgery followed by endocrine therapy.

In Edinburgh, small studies have been performed comparing neoadjuvant tamoxifen with aromatase inhibitors. Although patients were not randomised and the numbers were small, impressive results were achieved. Table 1 shows the number of patients who had reductions in tumour volume of more then 50% as assessed by ultrasound scan. As can be seen, 46% of patients treated with tamoxifen, 88% of patients treated with letrozole and 78% of patients treated with anastrozole had a reduction in tumour volume of greater than 50%. Of the whole group, only two patients progressed while on treatment.

There are no large randomised studies comparing neoadjuvant endocrine therapy with chemotherapy and little work has been done in this area since the patient populations who are most commonly treated with neoadjuvant chemotherapy tend to be premenopausal women with large ER negative tumours, in contrast those treated with endocrine therapy tend to be elderly postmenopausal women with ER positive tumours.

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Table 1
Median percentage in tumour volume as assessed by ultrasound

Drug	No.	No. >50% reduction	No. <50% reduction and <25% increase	No. >25% increase
Tamoxifen	65	30	34	1
Letrozole	24	21	2	1
Anastrozole	23	18	5	0

A potential problem with using tamoxifen as neoadjuvant therapy is the long time period required to reach steady state plasma levels—up to 5 weeks [11]. In contrast, the newer aromatase inhibitors build up rapidly reaching therapeutic concentrations within days.

1.2. Studies with letrozole

Initial studies performed in Edinburgh with letrozole, a highly selective aromatase inhibitor, suggested that there may be benefits of using aromatase inhibitors rather than tamoxifen in postmenopausal ER positive patients [12]. This led to randomised studies. The PO24 trial compared 4 months of neoadjuvant letrozole with tamoxifen in postmenopausal women with large breast cancers which required mastectomy or were locally advanced and inoperable and were ER or PgR positive [13]. This study demonstrated that letrozole achieved a significantly higher clinical response rate than tamoxifen (55% versus 36%; $P < 0.001$), enabling more patients treated with letrozole than with tamoxifen to undergo breast-conserving surgery (45% versus 35%; Table 2). Median time to response was 66 days in the letrozole group and 70 days in the tamoxifen group.

Modified WHO criteria was used to evaluate tumour response in the neoadjuvant setting as follows:

- *Partial response* (*PR*): Reduction in tumour size ≥50% from pre-treatment size.

Table 2
Primary and secondary efficacy end point results of trial P024 comparing 4 months of neoadjuvant letrozole vs. tamoxifen, in all study patients [12]

Efficacy end points	Letrozole (%) ($n = 154$)	Tamoxifen (%) ($n = 170$)	*P*-value
Primary end point			
Clinical response (palpation)	55	36	<0.001
Complete	10	4	
Partial	45	32	
Secondary end points			
Ultrasound response	35	25	0.042
Complete	3	1	
Partial	32	24	
Mammographic response	34	16	<0.001
Complete	4	0	
Partial	30	16	
Breast-conserving surgery	45	35	0.022

Table 3
Responses in trial P024 comparing 4 months of neoadjuvant letrozole vs. tamoxifen, relative to confirmed ER and/or PgR status [7]

Agent	Marker status	Response rate (%)	*P*-value
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	PgR negative	28	

ER, oestrogen receptor; PgR, progesterone receptor.

- *Minor response* (*MR*): Reduction in tumour size ≥25 and <50% from pre-treatment size.
- *No change* (*NC*): <25% decrease or <25% increase in tumour size from pre-treatment size.
- *Complete response* (*CR*): No measurable tumour.
- *Progressive disease* (*PD*): 25% or more increase in tumour size from pre-treatment size.

There were fewer responses demonstrated by ultrasound and mammography but responses were significantly more common with letrozole than tamoxifen whether assessed by ultrasound or mammography (Table 2). The only other factor besides treatment which influenced the likelihood of patients being suitable for breast-conserving surgery was tumour size at presentation with patients with T2 tumours being more likely to be candidates for breast-conserving surgery than larger tumours ($P = 0.0001$). In this randomized study, letrozole was at least as well tolerated as tamoxifen.

Tumour response in this study was related to ER and PgR status [5]. There were significantly more responses in patients subsequently confirmed to have ER positive tumours than in patients who on subsequent testing had ER negative tumours (Table 3). In each of the ER categories, response rates were higher for letrozole than tamoxifen. There appeared to be a particular difference in response rates in tumours that were ER positive and also over expressed erbB1 and/or erbB2 with an 88% response rate in this group for letrozole versus a 21% response rate to tamoxifen $P = 0.0004$ [5].

1.3. Studies with anastrozole

In Edinburgh, a series of 24 patients have been treated with neoadjuvant anastrozole [14]. These tumours have recently been stained for erbB2 and a correlation between the erbB2 status response and change in proliferation in hormone receptor has been undertaken [15]. Twenty-two patients had sufficient tumour in all their specimens to allow us to assess erbB2 status prior to treatment and also to study changes in proliferation as assessed by Ki67 antibody and PgR as assessed by the DAKO antibody. There were 6 erbB2 3+ tumours with the other 16 tumours being either

Table 4
Response rates and changes in Ki67 and PgR in 22 patients treated by 3 months of preoperative anastrozole subdivided according to erbB2 status

erbB2	No.	Clinical		Ultrasound		Median Ki67		Fall in PgR
		CR/PR	S.D.	CR/PR	S.D.	Pre	Post	
0/>1	16	15	1	10	6	23.5	>5	13/13*
>3	6	6	0	5	1	22.5	~7.5	3/4*

* Five patients PgR 0 on first biopsy: (a) $P = 0.017$ and (b) $P < 0.0001$.

negative or 1+. Comparison has been made between these two groups. There was no difference in clinical response between the two groups (Table 4), and initial proliferation and changes in proliferation and PgR receptor did not differ between the different groups. These data demonstrate anastrozole is clinically and biologically effective in erbB2 positive tumours.

An ongoing multicentre, randomised, double-blind clinical trial, Immediate Preoperative Arimidex Alone or in Combination with Tamoxifen (IMPACT) has now completed recruiting. It compares anastrozole 1 mg daily versus tamoxifen 20 mg daily versus anastrozole plus tamoxifen. Three hundred and thirty postmenopausal patients with ER and/or PgR positive breast cancers if large or operable, or potentially operable but locally advanced, have been recruited. In this study, treatment was for 3 months and patients providing they respond continue on the same endocrine treatment as adjuvant therapy for 5 years. Primary endpoints are objective tumour response rates with secondary endpoints being breast-conserving rate and assessment of key biological markers including proliferation, hormone receptors and apoptotic rate.

1.4. Newer Edinburgh studies

In the Edinburgh Breast Unit, we have now treated 83 patients with neoadjuvant letrozole [16]. We have correlated clinical and ultrasound responses and change in tumour volumes in these patients in relation to the ER ALLRED score. Sixty of the tumours were ER category 8 and 23 were category 6 or 7 (Table 5). Response rates were similar in ER categories 8 and 6 + 7 but there was a greater percentage reduction in tumour volume in patients whose tumours had the highest ER level. This difference was significant ($P < 0.05$).

1.5. Selection of patients for neoadjuvant therapy

The data outlined indicate that selection for neoadjuvant endocrine therapy should be based primarily on ER status and to a lesser extent, PgR status [5]. Although PO24 suggested that one of the differences between tamoxifen and letrozole is that patients with lower levels of ER are more likely to respond to letrozole than tamoxifen, the numbers in these categories was small and it remains our policy to treat patients who are fit for surgery with neoadjuvant endocrine therapy only if their ER ALLRED score is 6 or over because these are the women who are most likely to respond and gain a clinical benefit.

1.6. Duration of neoadjuvant therapy

Standard practice with neoadjuvant chemotherapy is to administer between three and six cycles prior to surgery, a time period felt sufficient to delineate responders from non-responders [17]. The optimal duration of neoadjuvant therapy has never been investigated in detail. One study at the Edinburgh Breast Unit gave neoadjuvant tamoxifen to 100 consecutive patients over the age of 70 with ER rich breast cancers (>20 fmol/mg cytosol protein) [18]. It demonstrated that after 3 months 72 had responded (based on a greater than 25% reduction in tumour volume by ultrasound) and one patient had progressive disease. The remaining 27 continued on tamoxifen for a further 3 months during which 18 patients' disease remained static, four responded but five progressed. From these data, it can be concluded that if patients are not responding by 3 months they are unlikely to respond and there is the concern that if left on tamoxifen alone the disease may progress. Three months therefore appears sufficient to demonstrate whether the tumour is responsive. Maximal response may however take considerably longer than 3 months and the optimal duration of therapy depends on initial tumour size and the aim of the neoadjuvant therapy. If the aim is to downstage the tumour to allow breast-conserving surgery, then this can be achieved in the majority of patients with 3–4 months treatment.

1.7. Response and downstaging in breast cancer

Response rates to preoperative chemotherapy are generally around 80% regardless of the regimen used [19]. In appropriately selected patients, neoadjuvant endocrine

Table 5
Response in 83 patients treated with 3 months of neoadjuvant letrozole subdivided according to ALLRED ER score

ALLRED ER score	No. of patients	No. of responders	% Response	Median % reduction in tumour volume	
				Clinical	USS
8	60	48	80	76*	67*
6 + 7	23	17	74	63	48

* $P < 0.05$.

Table 6
Local recurrences after neoadjuvant endocrine therapy followed by surgery with or without radiotherapy, in series of breast cancer patients at the Edinburgh Breast Unit

Agent	No. of patients	No. with no XRT[a]	Number with local recurrence	Number with XRT[b]	Number with local recurrence	Median follow-up (months)
Tamoxifen	47	13	4	34	0	84
Letrozole	34	10	4	24	1	70
Anastrozole	21	0	0	21	1	51
Exemestane	10	4	1	6	0	42
Total	112	27	9	85	2	62

[a] Number of patients who, following 3 months of neoadjuvant therapy, underwent breast-conserving surgery without local radiation therapy (XRT).
[b] Number of patients who, following neoadjuvant therapy, underwent both breast-conserving surgery and local radiotherapy.

therapy also produces response rates of up to 80% (Table 5). In the Milan study, 16% of 227 cases having breast-conserving surgery had evidence of multifocality tumour within the wide excision specimen with the frequency being highest in larger tumours [20]. Following neoadjuvant chemotherapy in the Royal Marsden series 28% of patients who underwent breast-conserving surgery had involved margins [21]. In a series of patients with locally advanced breast cancer treated with neoadjuvant chemotherapy prior to surgery, 62.5% of patients had multiple foci of tumour remaining after wide local excision [22]. This contrasts with our own experience of surgery after neoadjuvant endocrine therapy of 47 patients who initially were treated by breast-conserving surgery after treatment with neoadjuvant tamoxifen where in only one case was there an incomplete excision in a patient with invasive lobular cancer [23].

In a subsequent series treated with neoadjuvant aromatase inhibitors, 65 patients had breast-conserving surgery and only two of these had an incomplete excision. When the residual tumour was evaluated histologically, the nature of the response to neoadjuvant endocrine therapy was somewhat different in that the whole tumour appears to shrink concentrically whereas with chemotherapy the extent of disease was often noted by our pathologist to have remained unchanged while the cellularity of the tumour was usually markedly reduced.

1.8. Local recurrence following neoadjuvant endocrine therapy followed by breast-conserving surgery

Several studies have examined the rates of tumour recurrence following neoadjuvant therapy followed by surgery. Veronesi et al. reported 12 cases of local recurrence in 203 patients with a median follow-up of 3 years after preoperative chemotherapy [24]. This was considerably better than the 22% recurrence rate among the patients who underwent mastectomy because after chemotherapy they were unsuitable for breast-conserving surgery. In another trial, the recurrence rates were similar for patients receiving initial chemoendocrine therapy with recurrence rates of 3.5% compared with patients treated in the standard manner (surgery followed by systemic therapy) where the recurrence rate was 2.7% [25]. The only data available on local recurrence after breast-conserving surgery in patients treated with neoadjuvant endocrine therapy are presented in Table 6. These are data from Edinburgh and demonstrate that the overall recurrence rate without radiotherapy was 33% at 5 years [23]. If patients who did not have radiotherapy are excluded, only two patients from a total of 85 have developed a local recurrence at a median follow-up of 5 years. These results indicate that breast-conserving surgery followed by radiotherapy achieves satisfactory local disease control in patients downstaged by neoadjuvant endocrine therapy.

The population of patients who are treated with neoadjuvant endocrine therapy tend to be elderly and these patients can have significant comorbidity. For many of these patients despite locally advanced disease they will die from causes other than breast cancer. For this reason, it is difficult to compare long term survival of these patients with a series treated with adjuvant endocrine therapy.

Both disease free survival and overall survival have been reported to be similar in patients treated with neoadjuvant chemotherapy preoperatively and in patients treated with systemic therapy after surgery [26–29]. A recently presented trial comparing preoperative ER directed neoadjuvant versus adjuvant therapy was presented and showed no difference in survival for patients treated with neoadjuvant endocrine or chemotherapy compared with patients having initial surgery and follow-up systemic therapy [30].

2. Conclusion

Neoadjuvant endocrine therapy does appear to be effective. Reductions in tumour volume using primary endocrine therapy in ER and/or PgR positive tumours are similar to those reported with neoadjuvant chemotherapy. In contrast, toxicity is much lower with neoadjuvant endocrine therapy and it is extremely well tolerated, with very few patients having to discontinue therapy because of side effects.

From a surgical perspective, the ability to perform less extensive surgery is an advantage especially considering the comorbidity and overall general health of the group of patients who tend to be treated with neoadjuvant endocrine therapy. The currently available data suggests that breast-conserving surgery followed by radiotherapy

produces adequate local disease control in patients downstaged by neoadjuvant endocrine therapy.

The patients who are most likely to respond to neoadjuvant endocrine therapy are those who have higher levels of ER (ALLRED score 6 and above). Response rates to neoadjuvant therapy in postmenopausal women have been shown to be higher when using aromatase inhibitors than with tamoxifen. This may partly be due to the fact that aromatase inhibitors are effective in both erbB2 positive and negative cancers while tamoxifen is less effective in erbB2 positive tumours and that the aromatase inhibitors produce responses in tumours with lower levels of ER whereas tamoxifen does not.

Results of the currently ongoing trails using neoadjuvant endocrine therapy are awaited with interest.

References

[1] H. Bear, Indications for neoadjuvant therapy for breast cancer, Semin. Oncol. 25 (Suppl. 3) (1998) 3–12.

[2] M. Perloff, G.J. Lesnick, Chemotherapy before and after mastectomy in stage III breast cancer, Arch. Surg. 117 (1982) 879–881.

[3] P. Schick, J. Goodstein, J. Moor, J. Butler, K.L. Senter, Preoperative chemotherapy followed by mastectomy for locally advanced breast cancer, J. Surg. Oncol. 22 (1983) 278–282.

[4] G. Bonadonna, U. Veronesi, C. Brambilla, et al., Primary chemotherapy to avoid mastectomy in tumors with diameters of 3 cm or more, J. Natl. Cancer Inst. 82 (1990) 1539–1545.

[5] M.J. Ellis, A. Coop, B. Singh, et al., Letrozole is more effective neoadjuvant endocrine therapy than tamoxifen for erbB-1- and/or erbB-2-positive, oestrogen receptor-positive primary breast cancer: evidence from a phase III randomized trial, J. Clin. Oncol. 19 (2001) 3808–3816.

[6] G. Mustacchi, S. Milani, A. Pluchinotta, A. De Matteis, A. Rubagott, A. Perrota, Tamoxifen or surgery plus tamoxifen as primary treatment for elderly patients with operable breast cancer, GRETA Trial Anticancer Res. 14 (1994) 2197–2200.

[7] J.C. Gazet, H.T. Ford, R.C. Coombes, J.M. Bland, R. Sutcliffe, J. Quilliam, S. Lowndes, Prospective ramdomised trial of tamoxifen vs. surgery in elderly patients with breast cancer, Eur. J. Surg. Oncol. 20 (1994) 207–214.

[8] A.D. Van Dalsen, J. De Vries, Treatment of breast cancer in elderly patients, J. Surg. Oncol. 60 (1995) 80–82.

[9] T. Bates, D.L. Riley, J. Houghton, L. Fallowfield, M. Baum, Breast cancer in elderly women: a Cancer Research Campaign trial comparing treatment with tamoxifen and optimal surgery with tamoxifen alone, Br. J. Surg. 78 (1991) 591–594.

[10] F.S. Kenny, J.F.R. Robertson, I.O. Ellis, C.W. Elston, R.W. Blamey, Primary tamoxifen versus mastectomy and adjuvant tamoxifen in fit elderly patient with operable breast cancer of high ER content, Breast 8 (1999) 216.

[11] J.M. Dixon, Neoadjuvant Endocrine Therapy. Breast Cancer Diagnosis and Management, Elsevier, London, 2000 (Chapter 38).

[12] J.M. Dixon, C.D.B. Love, C.O.C. Bellamy, D.A. Cameron, R.C.F. Leonard, H. Smith, W.R. Miller, Letrozole as primary medical therapy for locally advanced and large operable breast cancer, Breast Cancer Res. Treat. 66 (3) (2001) 191–199.

[13] W. Eiermann, S. Paepke, J. Appfelstaedt, et al., Preoperative treatment of postmenopausal breast cancer patients with letrozole: a randomized double-blind multicenter study, Ann. Oncol. 12 (2001) 1527–1532.

[14] J.M. Dixon, L. Renshaw, C. Bellamy, M. Stuart, G. Hoctin-Boes, W.R. Miller, The effects of neoadjuvant anastrozole (Arimidex) on tumor volume in postmenopausal women with breast cancer: a randomized, double-blind, single-center study, Clin. Cancer Res. 6 (2000) 2229–2235.

[15] J.M. Dixon, J. Jackson, M. Hills, L. Renshaw, D.A. Cameron, T.J. Anderson, W.R. Miller, M. Dowsett, Anastrozole demonstrates clinical and biological effectiveness in erbB2 ER positive breast cancers, Breast Cancer Res Treat., in press.

[16] J.M. Dixon, J. Jackson, L. Renshaw, D.A. Cameron, W.R. Miller, Neoadjuvant letrozole: the Edinburgh experience, Breast Cancer Res. Treat., in press.

[17] J.H. Goldie, Scientific basis for adjuvant and primary (neoadjuvant) chemotherapy, Semin. Oncol. 14 (1987) 1.

[18] J.M. Dixon, Neoadjuvant therapy: surgical perspectives, in: W.R. Miller, J.N. Ingle (Eds.), Endocrine Therapy in Breast Cancer, Marcel Dekker, New York, 2002, pp. 197–212.

[19] G. Bonadonna, P. Valagussa, C. Brambilla, et al., Adjuvant and neoadjuvant treatment of breast cancer with chemotherapy and/or endocrine therapy, Semin. Oncol. 15 (1991) 515–524.

[20] U. Veronesi, G. Bonadonna, S. Zurrida, et al., Conservation surgery after primary chemotherapy in large carcinomas of the breast, Ann. Surg. 222 (1995) 612–618.

[21] L. Mauriac, G. MacGrogan, A. Avril, et al., on behalf of Institute Bergonie Bordeaux Groupe Sein (IBBGS). Neoadjuvant chemotherapy for operable breast carcinoma larger than 3 cm: a unicentre randomised trial with a 124-month follow up, Ann. Oncol. 10 (1990) 47–52.

[22] M. Moneer, M. El-Didi, H. Khaled, Breast conservation surgery: is it appropriate for locally advanced breast cancer following downstaging by neoadjuvant chemotherapy—a pathological assessment, The Breast 8 (1999) 315–319.

[23] J.M. Dixon, T.J. Anderson, W.R. Miller, Neoadjuvant endocrine therapy of breast cancer: a surgical perspective, Eur. J. Cancer, in press.

[24] U. Veronesi, G. Bonadonna, S. Zurrida, S. Andreola, F. Rilke, R. Raselli, M. Merson, V. Sacchini, R. Agresti, Conservation surgery after primary chemotherapy in large carcinomas of the breast, Ann. Surg. 222 (1995) 612–618.

[25] A. Makris, T.J. Powles, S.E. Ashley, et al., A reduction in the requirements for mastectomy in a randomised trial of neoadjuvant chemoendocrine therapy in primary breast cancer, Ann. Oncol. 9 (1998) 1179–1184.

[26] J.M. Kurtz, Should surgery remain the initial treatment of operable breast cancer? Eur. J. Cancer 27 (12) (1991) 1539–1542.

[27] B. Fisher, J. Bryant, N. Womark, E. Mamounas, A. Brown, E.R. Fisher, D.L. Wickerham, M. Begovic, A. DeCillis, A. Robidoux, R.G. Margolese, A.B. Cruz Jr., J.L. Hoehn, A.W. Lees, N.V. Dimitrov, H.D. Bear, Effect of preoperative chemotherapy on the outcome of women with operable breast cancer, J. Clin. Oncol. 16 (8) (1998) 2672–2678.

[28] L. Mauriac, M. Durand, A. Avril, J.M. Dilhuydy, Effects of primary chemotherapy in conservative treatment of breast cancer patients with operable tumours larger than 3 cm. Results of a randomised trial in a single centre, Ann. Oncol. 2 (5) (1991) 347–354.

[29] V.F. Semiglazov, E.E. Topuzov, J.L. Bavli, V.M. Moiseyendo, O.A. Ivanova, I.K. Seleznev, A.A. Orlov, N.Y. Barash, O.M. Golubeva, O.F. Chepic, Primary (neoadjuvant) chemotherapy and radiotherapy compared with primary radiotherapy alone in stage IIb–IIIa breast cancer, Ann. Oncol. 5 (7) (1994) 591–595.

[30] D.A. Cameron, W. Jack, P. Forouhi, J. Keen, J.M. Dixon, R.C.F. Leonard, U. Chetty, Oestrogen receptor directed primary systemic therapy: a randomised trial compared with conventional therapy in operable breast cancer, Breast Cancer Res. Treat. 76 (Suppl.) (2002) S5. Abstract 157.

Expert Opinion

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The therapeutic potential of aromatase inhibitors

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The third generation aromatase inhibitors are both remarkably potent and specific endocrine agents inhibiting aromatase activity and reducing circulating oestrogen levels in postmenopausal women to levels never previously seen. Their therapeutic potential is consequently much greater than the earlier prototype drugs. Their excellent side-effect profile also allows for potential wider indications in the treatment of oestrogen-related diseases, including breast cancer. It still remains to determine whether their potent endocrine effects translate into increased therapeutic benefit. In advanced breast cancer, aromatase inhibitors have been shown to have improved efficacy and toxicity profiles when compared with progestins, aminoglutethimide and tamoxifen. Aromatase inhibitors have also been used in the neoadjuvant setting, where they have been shown to achieve higher response rates than tamoxifen and to be more successful at downstaging tumours. Early results comparing an aromatase inhibitor with tamoxifen in the adjuvant setting in early breast cancer show anastrozole to be superior to tamoxifen in terms of both disease-free survival and a lower incidence of new contralateral tumours. There was also a more favourable side-effect profile, which has implications for potential future prophylactic treatment. Additionally, since aromatase inhibitors have different mechanisms of action, unlike antioestrogens, they may be particularly useful as chemopreventive agents if oestrogens are themselves genotoxic. Aromatase inhibitors have been used to date almost exclusively in postmenopausal women. The potential of combining them with luteinising hormone-releasing hormone analogues allows the possibility of treating premenopausal women with either oestrogen receptor-positive breast cancer or benign conditions such as cyclical breast pain, fibroadenomata, recurrent cystic disease or endometriosis. There is also the potential for their use in men with conditions such as gynaecomastia or prostate cancer. These new generation aromatase inhibitors may well have an increasing role in the future management of a number of conditions in addition to breast cancer.

Keywords: aromatase inhibitors, breast cancer, endocrine, oestrogen, therapeutic

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1. Introduction

Whilst oestrogen is classically regarded as the female sex hormone, being primarily responsible for sexual development in women (most notably the functional regulation of the uterus, breast and ovary), its actions stretch beyond this. For example, oestrogen plays a crucial role in normal metabolism of bone and lipids [1]. Furthermore, it is increasingly recognised to play a role in the natural history of diseases associated with hormone irregularities including neoplasia of the breast, uterus and ovary [2].

Consequently, the concept of interfering with the synthesis and/or mechanism of action of oestrogen is an attractive option to control hormone-related disease proc-

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esses. In terms of biosynthesis, oestrogens are the end point in a sequence of reactions in which other important hormones such as progestins and androgens are intermediaries. The most specific method by which to inhibit oestrogen formation (and thereby avoid the side effects of interfering with the action of other hormone classes) is to block the last step in the biosynthetic cascade. This is the conversion of androgens, such as androstenedione and testosterone, into oestrogens, such as estrone and oestradiol. Since the reaction renders the steroid molecule aromatic, the enzyme is known as 'aromatase'.

Drugs which inhibit aromatase are not new; indeed the prototype drug aminoglutethimide was first used to treat breast cancer over 30 years ago [3]. However, the agent was not specific, inhibiting the biosynthesis of other steroid classes (and required the concomitant administration of corticoid replacement therapy); neither was aminoglutethimide particularly potent, the standard dose being 1 g/day (250 mg q.i.d.) [4]. However, in the intervening years, rational drug development has seen the evolution of agents which inhibit aromatase with remarkable potency and specificity. Consequently, they have a therapeutic potential which far exceeds that of the prototype drugs. This article will review the endocrine profile of the latest generation of aromatase inhibitors (AIs) and consider their potential use in the treatment of oestrogen-related diseases, most particularly breast cancer.

2. Classification of aromatase inhibitors

Inhibitors of aromatase may be subdivided into two main classes (**Figure 1**). Type I inhibitors interact with the substrate-binding sites of the enzyme. In structure, most are androgens (**Figure 2**); as a consequence they may have hormonal activity [5]. However, the inhibition may be mechanism-based in that the drugs, when bound to the catalytic site of the enzyme, are metabolised to intermediates which attach irreversibly to the active site, thereby blocking activity [6]. Such inhibitors would be expected to be particularly specific, only inhibiting molecules for which they are substrates. Because of the irreversible nature of the inhibition, the enzyme remains inactive even after the drug is cleared from the circulation. These inhibitors have, therefore, been marketed as 'inactivators' [7].

Type II inhibitors interact with the haem moiety of the cytochrome P450 (CYP450) prosthetic group of the aromatase molecule (**Figure 1**). These inhibitors are imidazoles and triazoles (**Figure 2**). Early Type II drugs had poor specificity because they interacted with the CYP450 group in other enzymes [8]. However, the amino acid sequence of CYP450 aromatase is distinct from other members of the CYP450 family [9] and the latest generation of drugs have complete selectivity towards CYP450 aromatase, thus allowing specific inhibition. In contrast to Type I agents, Type II inhibitors are generally reversible and oestrogen blockade is dependent upon continued presence of the drug.

The first AIs were used without the knowledge that they had antiaromatase properties [10-12]. These drugs included aminoglutethimide and testolactone (**Table 1**). Additionally, the drugs lacked specificity and produced side effects unconnected with effects on aromatase. Second generation drugs were considerably more potent but either had poor pharmocokinetics when given orally (formestane) [13] or affected other steroidogenic enzymes in addition to aromatase (fadrozole) [14].

Table 1. Anti-aromatase agents by generation.

Generation	Type I agents (Steroidal inactivators)	Type II agents (Non-steroidal inhibitors)
First	Testolactone	Aminoglutethimide
Second	Formestane	Fadrozole
Third	Exemestane	Anastrozole Letrozole

As reviewed below, the latest third generation inhibitors (anastrozole, letrozole and exemestane) are exceptionally potent and specific, blocking aromatase at nanomolar concentrations [15] and being capable of reducing endogenous oestrogen in postmenopausal women to undetectable levels whilst having no discernable effects on other hormone classes [16].

3. *In vitro* studies

Placental microsomes possess high aromatase activity and have been classically used to assess the ability of drugs to inhibit oestrogen biosynthesis. Results relating to aminoglutethimide, anastrozole, letrozole, exemestane and formestane are presented in **Figure 3A**. All agents inhibited aromatase activity in a dose-related manner, but whereas aminoglutethimide needed to be used at micromolar concentrations, the newer generation of inhibitors were magnitudes of order more potent, being effective at nanomolar concentrations.

Although placental microsomes are widely used as a screen for antiaromatase agents, the level of aromatase activity does not reflect that in peripheral tissues, which is minute in comparison. Since AIs are currently reserved for postmenopausal patients, it is more appropriate to screen inhibitors against peripheral tissues, which are the primary site of oestrogen biosynthesis in these patients. About 70% of breast cancers display aromatase activity when incubated *in vitro* as particulate fractions [17]. Therefore, such tumour homogenates are useful test systems, as is shown in **Figure 3B**. Again, the newer generation of agents produce dose-related inhibition at nanomolar concentration, whereas aminoglutethimide requires micromolar levels. Concentrations which produce 50% inhibition of activity (IC_{50}) are summarised in **Table 2**. It can be seen that:

- the Type II inhibitors (anastrozole and letrozole) are more potent than the Type I inhibitors (formestane and exemestane)
- letrozole is more powerful than anastrozole, and exemestane is more powerful than formestane
- the third generation inhibitors are at least 150-fold more active than aminoglutethimide in this test system

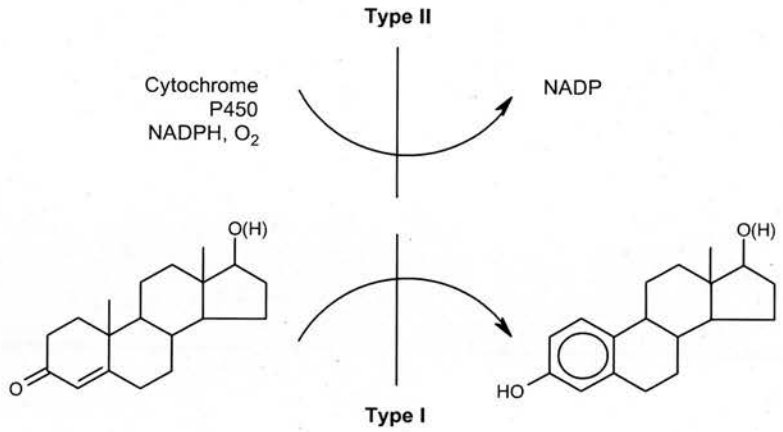

Figure 1. Types of anti-aromatase agents.

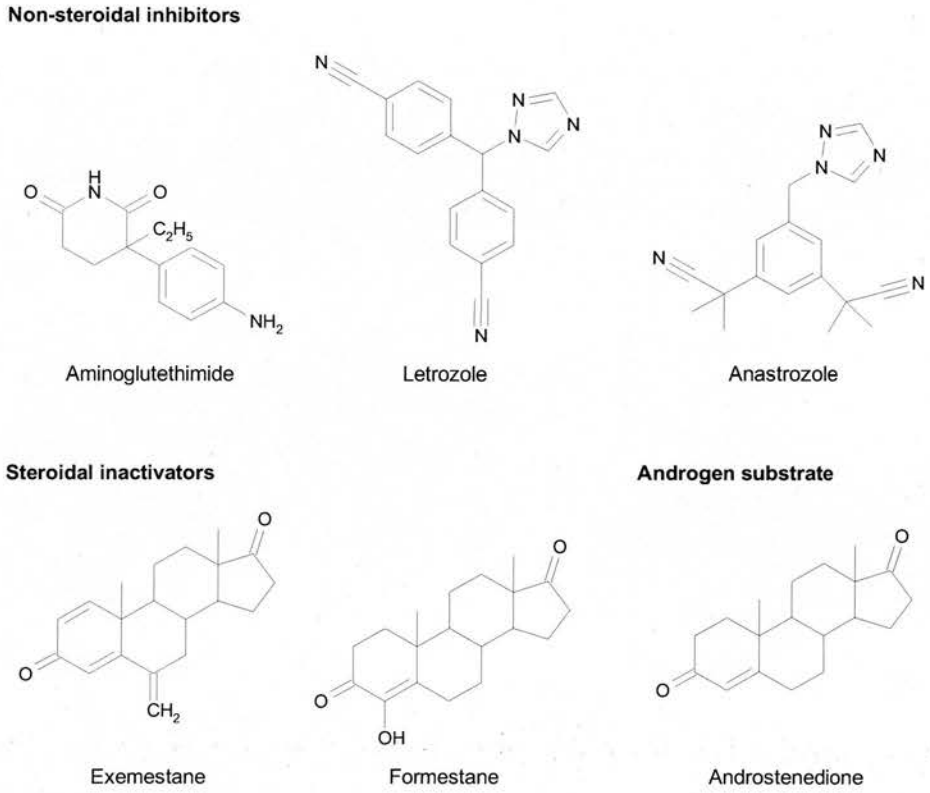

Figure 2. Differences in structure of anti-aromatase agents.

Whilst disrupted cell preparations are useful model systems, they do not take into account other factors such as cellular uptake, intracellular sequestration and metabolism of drugs. It is therefore advisable to include whole-cell systems, such as cultured fibroblasts from breast adipose tissue, in any comparison. Such observations are shown in **Figure 4A**. The results are consistent with other whole cell systems [18] in that letrozole appears to increase differentially in potency as compared with other Type II inhibitors, such as aminoglutethimide and anastrozole. Interestingly, exemestane also appears more active in whole-cell cultures than in disrupted cell preparations.

Cultured fibroblasts can also be used to demonstrate the difference in mechanism of action between irreversible Type I inhibitors and reversible Type II agents. These fibroblasts can be preincubated with inhibitors which are then removed before

Table 2. Inhibition of aromatase activity in whole-cell and disrupted-cell preparation.

Compound	Placental microsomes		Breast cancer homogenates		Mammary fibroblast cultures	
	IC_{50} (nM)	Relative potency	IC_{50} (nM)	Relative potency	IC_{50} (nM)	Relative potency
Aminoglutethimide	3000	1	4500	1	8000	1
Anastrozole	12	250	10	450	14	570
Letrozole	12	250	2.5	1800	0.8	10,000
Formestane	50	60	30	150	45	180
Exemestane	50	60	15	300	5	1600

IC_{50}: 50% Inhibitory concentration.

assay for aromatase. If the inhibitor has an irreversible action, aromatase activity will remain inhibited, but those with reversible characteristics will be ineffective. The results in **Figure 4B** suggest that the Type II agents – aminoglutethimide, anastrozole and letrozole – are reversible, whereas exemestane and formestane are irreversible. Interestingly, at least at one concentration, all Type II inhibitors produced a paradoxical increase in aromatase activity compared with control. Enhanced aromatase activity is also produced by aminoglutethimide in other systems [19]. This increased activity results from enhanced transcription of the aromatase gene [20] and stabilisation of the aromatase protein [21]. These effects probably account for the increased levels of *ex vivo* aromatase activity following aminoglutethimide treatment of patients with breast cancer [22]. It has been postulated that increased levels of aromatase protein following chronic treatment with Type II inhibitors could lead to break-through of oestrogen synthesis and renewed tumour growth [23].

Finally, *in vitro* screens on other closely related steroid-metabolising enzymes have demonstrated the exquisite selectivity of the newer inhibitors. This specificity is illustrated for exemestane in **Table 3**. The drug only affected other steroid hydroxylases at concentrations that were at least 2800-fold higher than those influencing aromatase.

Table 3. Inhibition of hydroxylases by exemestane.

Enzyme	IC_{50} (nM)	Relative specificity
Aromatase	25.4	1
21-Hydroxylase	> 100,000	> 3937
11ß-Hydroxylase	72,500	2854
18-Hydroxylase	84,500	3327
C_{20-22}-lyase	> 100,000	> 3937

The IC_{50} values of exemestane for aromatase and other steroid hydroxylases. Unpublished data from di Salle E, personal communication.
IC_{50}: 50% Inhibitory concentration.

4. *In vivo* studies

Prior to giving drugs to postmenopausal women with breast cancer as treatment, studies have been performed in which the effects on the agents have been monitored *in vivo* on whole body peripheral aromatase and circulating levels of oestrogen [24,25].

To measure peripheral aromatase, radioactive androgen precursors were given and the conversion to oestrogens assayed by measuring the radioactivity in oestrogens purified from either urine or plasma. Measurements before and after 2 – 3 months of treatment with daily milligram amounts of the novel AIs (letrozole, anastrozole and exemestane) indicate that these newer drugs are able to inhibit peripheral aromatase by > 97% [24]. This is a substantial improvement over aminoglutethimide, which, even when given in gram doses, inhibits peripheral aromatase by only 90% [24]. (Whilst the difference between 90 and 97% might not seem large, the improvement is probably better appreciated by considering the residual aromatase which is < 3% with the newer inhibitors compared with 10% with aminoglutethimide.)

As a consequence of this inhibition, circulating levels of oestrogen in postmenopausal women fall to levels which are at the level of detection of current assays [26,27]. This degree of suppression is greater than that produced by earlier generation inhibitors. For example, incremental reductions in oestrogen levels can be seen when switching patients relapsing on aminoglutethimide onto exemestane [28]. Effects are achieved without influences on other circulating hormones [26,27,29] and can be contrasted with those of aminoglutethimide which, because of its suppressive effects on corticosteroid synthesis, require corticoid replacement [30].

Because of this extraordinary potency and specificity for oestrogen suppression, the third generation AIs produce endocrine profiles in postmenopausal women that have rarely been observed previously. An expectation has thereby been created that these characteristics might be associated with increased therapeutic benefits, as outlined below.

5. Advanced breast cancer

For most women with advanced metastatic breast cancer, the outlook is comparatively bleak and the majority will ultimately die of their disease. Currently, the major realistic objectives of treatment are therefore to slow progression and to palliate symptoms rather than effect a cure. What might reasonably be expected of the newer generation of AIs in this setting? Their

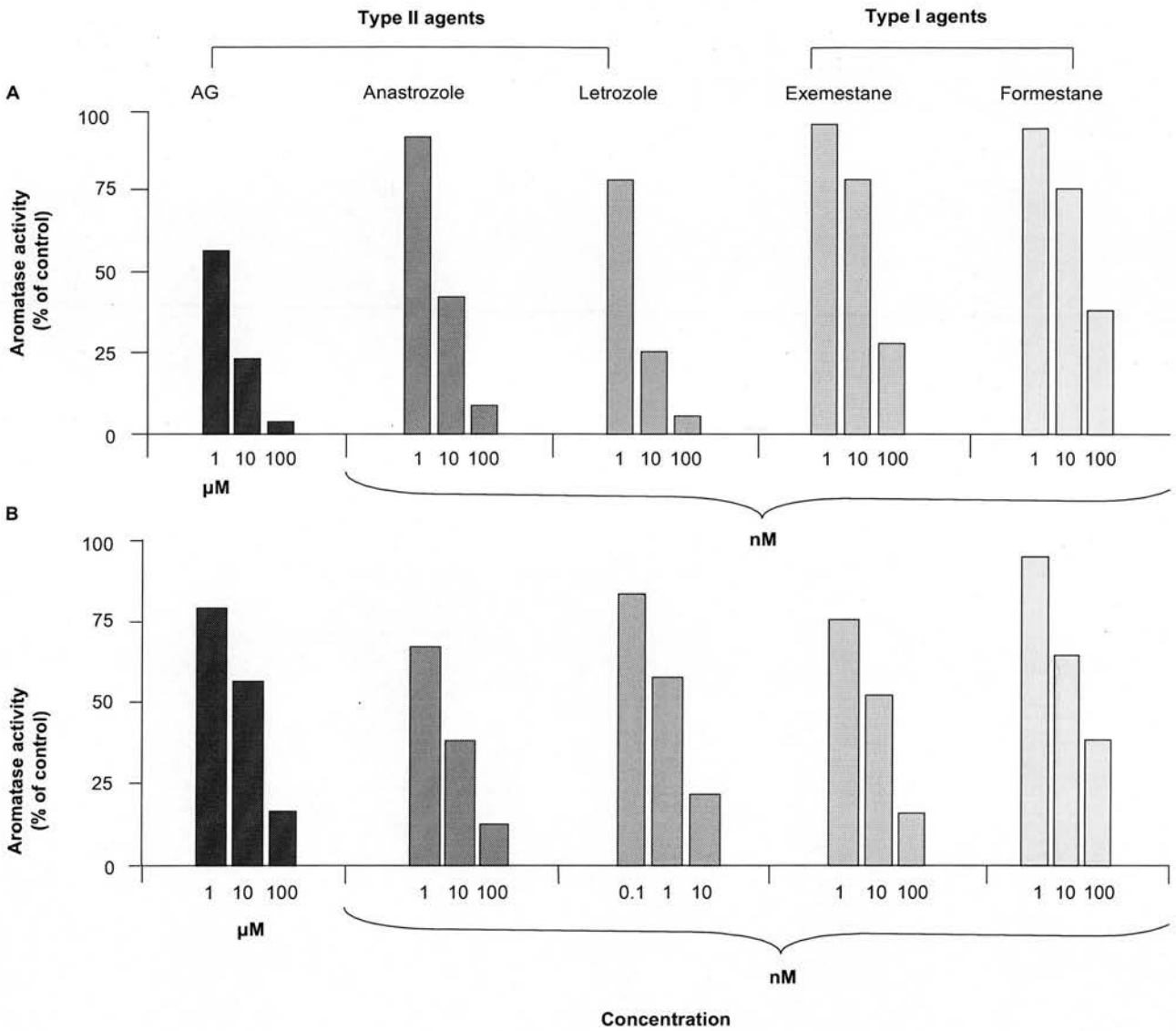

Figure 3. Effects of anti-aromatase agents on aromatase in breast cancer homogenates.
AG: Aminoglutethimide.

characteristics are the specific reduction of oestrogen concentrations in postmenopausal women to levels which have never been consistently achieved previously. The consequence of this, in the setting of tumour hormone dependence, would be:

- cancers which require oestrogen for their growth would be more efficiently deprived of hormones
- other tumours which are able to grow with the support of comparatively small amounts of oestrogen and appear resistant to less efficient hormone therapy, might respond to more effective drugs

In comparison to earlier AIs, the clinical expectations are, therefore, that the new generation drugs will produce increased response rates and second-line responses in patients relapsing on first-line inhibitors, as well as longer duration of response (such that some patients may die of non-cancer-related causes, i.e., a potential increase in cure rates). However, because the inhibitors are reducing oestrogen levels to extraordinary low levels, further responses with hormonal agents at relapse will be less likely. It is also worth making the comparison with tamoxifen, which until recently was established as first-line therapy for women with hormone sensitive disease. Although both tamoxifen and AIs have in common oestrogen deprivation as their end point, the drugs differ markedly in their mechanism of action. AIs reduce oestrogen levels, whereas intervention with tamoxifen blockades oestrogen action whilst leaving

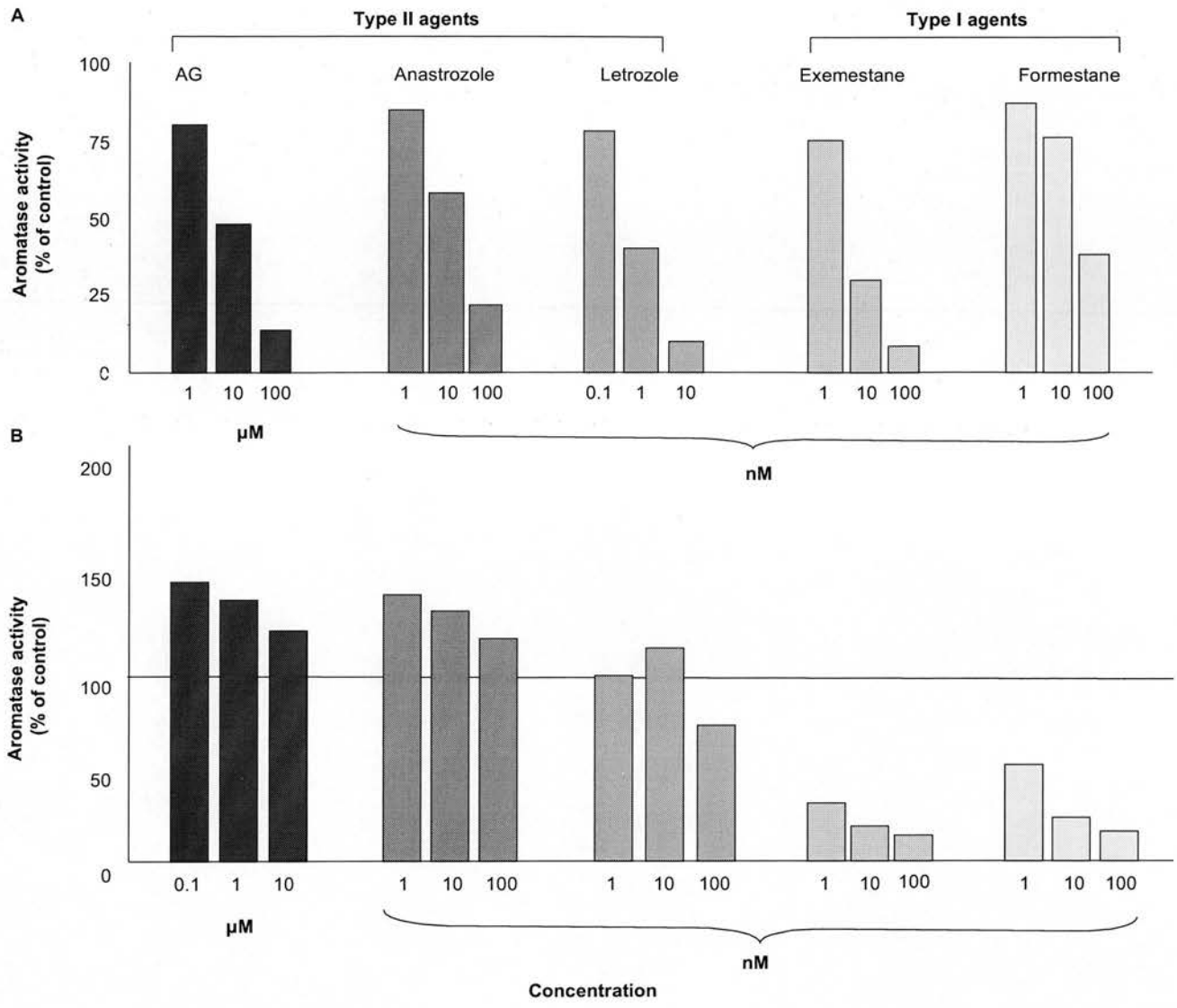

Figure 4. Inhibition of aromatase activity in fibroblasts.
AG: Aminoglutethimide.

levels of the hormone unchanged (or even raised) [31]. Tamoxifen may also have oestrogen agonist activity which may reduce its effectiveness and lead to resistance [32]. The molecular changes which occur in tumours following treatment with tamoxifen are also clearly different from those resulting from therapy with AIs [33]. With these differences in mechanism of action, it might be expected that AIs may:

- be effective in tamoxifen-resistant tumours
- produce increased response rates (if oestrogen suppression is more effective than oestrogen antagonism)
- be particularly effective in different cohorts compared with tamoxifen within groups of hormone-sensitive tumours
- produce responses more quickly (AIs reduce oestrogen levels rapidly [29,34], whereas the concentrations of tamoxifen for effective oestrogen blockade accumulate relatively slowly) [35].

Whilst AIs have toxicities, their short-term side effects are comparatively minimal and concerns are largely related to chronic exposure (see Section 7). It might therefore be predicted that AIs will be an acceptable form of treatment for advanced disease. The evidence in support (or otherwise) of the above theoretical considerations is accumulating from completed and ongoing clinical trials and these will now be reviewed.

Initially, studies were conducted in the second-line setting in which AIs were compared with either progestins or early generation inhibitors (e.g., aminogluthethimide) in patients resistant to or relapsing on tamoxifen. A summary of the results from

Table 4. Second-line therapy with aromatase inhibitors after failure of tamoxifen in advanced breast cancer [36-41].

	ANA versus MA (1/160 mg)	LET versus MA (2.5/160 mg)	EXE versus MA (25/160 mg)	LET versus AG (2.5/50 mg)	LET versus MA (2.5/160 mg)
No of patients	263/253	174/189	336/403	185/178	199/201
Response rate, complete + partial response (%)	12.6/12.2	24/16*	15/12.4	19.5/12.3	16.1/14.9
Complete response + partial response + stable disease > 24 weeks (%)	42.2/40.3	35/32	37.4/34.6	36.3/29.3	26.7/23.4
Median TTP (months)		5.6/5.5	4.7/3.8*	3.4/3.2*	3/3
Median TTF (months)		5.1/3.9*	3.8/3.7*	3/3*	3/3
Median OS (months)	27/23*	25/22	NR/28.4*	28/20*	29/26

*Significant results.

AG: Aminoglutethimide; ANA: Anastrozole; EXE: Exemesrane; LET: Letrozole; MA: Megestrol acetate; NR: Not reached; OS: Overall survival; TTF: Time to treatment failure; TTP: Time to tumour progression.

these trials is shown in Table 4. Most trials concluded that in comparison with other earlier endocrine agents, the third generation AIs had superior efficacy and/or toxicity profiles and none of them were shown to be significantly inferior to the comparator in any end point of efficacy [36-41]. When many of these initial studies were performed, the oestrogen receptor (ER) and progesterone receptor (PgR) status for all patients enrolled was often not known. For example, between 15 and 20% of patients enrolled into the Letrozole versus Megestrol Acetate study had unknown ER and PgR status [41]. In the FEM-INT.01 head-to-head trial of letrozole versus anastrozole, 713 patients who had progressed on antioestrogen therapy were enrolled. In this study, which involved 112 centres in 19 countries, only 48% of the patient population had confirmed hormone receptor-positive status [42]. Since it has now been established that AIs are only effective in patients with hormone receptor-positive disease, some of the discrepancies that have been observed between studies may be explained by the heterogeneity of the patient population.

Several small Phase II trials have evaluated the role of AIs, particularly exemestane, after failure of two previous hormonal therapies (i.e., tamoxifen plus another agent) [43-45]. Although objective response rates were low (5 – 13%), they were similar to response rates in Phase III studies of patients treated with third generation AIs after failure of only one previous hormonal therapy [39,46]. In advanced disease there appears to be a lack of cross-resistance between tamoxifen and AIs and inactivators, which implies that sequential treatment may also be beneficial.

As a result of these favourable findings, both anastrozole and letrozole have recently been compared directly with tamoxifen as first-line treatment for advanced breast cancer. A study comparing exemestane with tamoxifen has recently completed recruiting patients, but results are not yet available.

The PO25 trial compared letrozole with tamoxifen in 939 patients for the first-line treatment of locally advanced or metastatic postmenopausal breast cancer [47,48]. Letrozole was shown to have a superior time to progression (9.4 months compared with 6 months for tamoxifen ($p < 0.0001$) and a significantly greater objective response rate (32% on letrozole, 21% on tamoxifen; $p = 0.0002$). However, there was no statistically significant difference in survival between the two groups. In this study patients were allowed to cross over from one drug to another and it may be that letrozole is superior in the second-line setting, especially as survival at early time points (i.e., up to 2 years) was significantly higher in the letrozole group. The side-effect profile in both groups was similar in this study.

Two randomised, double-blind, multi-centre Phase III trials were published in 2000 comparing anastrozole with tamoxifen as first-line therapy for advanced breast cancer in postmenopausal women [49-51]. The North American study showed a clinical benefit in favour of anastrozole with a median time to progression of 11.1 months for patients treated with anastrozole compared to 5.6 months for tamoxifen ($p = 0.0098$) [49,50]. However, this was not reflected in the other larger study, conducted in Europe, Australia, New Zealand, South America and South Africa [51]. An important difference between the trials was that 89% of the patients in the North American trial were known to be receptor positive compared with only 45% in the other trial. Again, currently available data show no difference in survival between patients treated with anastrozole and tamoxifen.

In summary, third generation AIs are now established as standard second-line treatment of advanced ER-positive breast cancer after tamoxifen because of improved efficacy and reduced adverse effects [52]. Trials in metastatic breast cancer have shown superior efficacy and toxicity profiles when comparing AIs and tamoxifen as first-line endocrine therapy. This has resulted in the increasing use of AIs as first-line treatment for metastatic ER-positive breast cancer in postmenopausal women. It will be necessary to run head-to-head trials of the available AIs to determine which has the best profile in terms of efficacy and toxicity.

6. Neoadjuvant therapy

Neoadjuvant therapy is most frequently given to patients with large primary tumours with the intent of shrinking the cancers so that inoperable lesions become operable. More conservative surgery may also be performed. Elderly or infirm patients may avoid surgery altogether. In this respect an effective and rapid therapy is particularly attractive since early tumour shrinkage would reduce time to surgery and provide psychological reassurance of regressing disease. The characteristics considered above for advanced disease suggest that AIs may have advantages over other endocrine therapies in this setting and should be associated with better and quicker response rates and decreased needs for surgery. The clinical trial evidence for this, together with the associated research data (neoadjuvant protocols have provided unique opportunities for research since the primary tumour is accessible for biopsy and assessment of response), is summarised below.

Small non-randomised neoadjuvant endocrine studies have been performed in the Edinburgh Breast Unit using tamoxifen, anastrozole, exemestane and letrozole. Comparing letrozole with tamoxifen showed a significant difference in tumour response rates (letrozole 81%, tamoxifen 48%) over a 3-month period [51]. Comparable results were achieved in a similar study using anastrozole [53]. Significant tumour volume reductions were achieved as shown in **Table 5**. This allowed a large percentage of patients who would have required a mastectomy pretreatment to have a wide local excision as their breast surgery (see **Table 6**).

The optimum duration of neoadjuvant endocrine therapy has not yet been established [54]. A small series of 94 elderly patients with ER-positive tumours (> 20 fmol/mg of cytosolic protein) were treated with neoadjuvant tamoxifen in Edinburgh (Keen J, MD thesis, Edinburgh, 1996). After 3 months of treatment, 69 patients had responded (> 10% tumour volume reduction on ultrasound scan) and seven patients had progressing disease. At this point, 26 patients elected to continue on tamoxifen for a further 3 months (20 responders and 6 non-responders). Of the 20 responders, 14 continued to show tumour shrinkage, one remained static, but five tumours which had initially responded showed evidence of disease progression. Of the six tumours that had shown no response at 3 months, two increased in size by 5 months and the remaining four did not change in volume. This data suggests that continuing patients on neoadjuvant therapy for > 3 months carries a significant risk of progression in tumours which initially respond and that if patients have not responded by 3 months they are unlikely to respond.

These studies have also yielded informative results regarding the effects of aromatase inhibition on tumour morphology and histology. It is clear that AIs consistently reduce proliferation in ER-rich tumours and reduce tumour grade by decreasing mitotic figures. Additionally, immunohistochemical staining for steroid receptors showed that letrozole and anastrozole, whilst having little effect on ER, produced marked reduction in the expression of PgR. These observations would be consistent with oestrogen deprivation and contrast with the effect of tamoxifen, which markedly reduces ER expression but may actually increase that of the PgR [33,55].

These initial non-randomised studies have provided the impetus for larger multi-centre randomised trials including the PO24 and Immediate Preoperative Arimidex Compared to Tamoxifen (IMPACT) trials.

The PO24 trial randomised 324 postmenopausal women with receptor-positive breast cancer to receive 4 months of neoadjuvant letrozole or tamoxifen [56]. Letrozole achieved a significantly higher clinical response rate than tamoxifen (55 versus 36%; $p < 0.0001$). This allowed more letrozole-treated patients than tamoxifen-treated patients to undergo breast-conserving surgery (**Table 7**). The study also generated some interesting observations regarding tumour biology. For example, tumours with low ER responded to letrozole, but not to tamoxifen, and ER-positive, cerbB-1/2-positive tumours had an 88% response rate with letrozole but only 21% with tamoxifen ($p = 0.0004$).

The IMPACT trial has recently completed recruiting patients. It randomised 330 patients to receive anastrozole, tamoxifen or a combination of both for 3 months before surgery and then as adjuvant therapy for 5 years. It is a multicentre, randomised, double-blind trial involving postmenopausal patients with ER-positive and/or PgR-positive breast cancer that is large and potentially operable. As its primary efficacy end points are objective tumour response at 3 months and secondary end points include breast conservation rate, biological markers, for example, ER, PgR, Ki67 and safety, it will generate important data on clinical response and tumour biology.

7. Early breast cancer

In the treatment of early breast cancer, toxicity of therapies becomes a more important consideration. Many women following surgery appear free of disease but require long-term systemic therapy for occult micrometastatic disease. So what is the potential of AIs in this setting? More effective hormone suppression might be expected to:

- increase disease-free interval
- reduce relapse rates, particularly in the medium term (in the short term, most relapses may be expected to come from the cohort of inherently aggressive, hormone-insensitive tumours that will not respond to any form of endocrine therapy; in the long term, recurrences may consist of hormone-insensitive outgrowths from more benign tumours)
- lessen the chance of recurrent disease responding to other hormone therapies
- be associated with problems emanating from long-term oestrogen deprivation, in particular bone loss and unfavourable changes in lipid profiles

Table 5. Tumour response in series of patients with locally advanced breast cancer who received neoadjuvant endocrine therapy in the Edinburgh Breast Unit.*

Agent	No. of patients	Patients with > 50% reduction, n (%)	Patients with < 50% reduction or < 25% increase, n (%)	Patients with > 25% increase, n (%)
Tamoxifen	65	30 (46)	34 (52)	1 (2)
Letrozole	36	32 (89)	3 (8)	1 (3)
Anastrozole	23	18 (78)	5 (13)	0
Exemestane	12	10 (83)	2 (17)	0

*Tumour volume changes (reduction or increase) were assessed by ultrasound measurements during the 3-month treatment period.

Table 6. Patients with locally advanced breast cancer requiring mastectomy before and after neoadjuvant endocrine therapy, in studies performed in the Edinburgh Breast Unit.

Agent	No. of patients	Number initially requiring mastectomy	Number requiring mastectomy after treatment	Conversion rate (%)*
Tamoxifen	65	41	15	63
Letrozole	36	24	2	93
Anastrozole	24[‡]	19	2	89
Exemestane	12	10	2	80

*Percentage of patients initially considered only for mastectomy who underwent breast-conserving surgery following treatment. [‡]Includes one patient who did not complete full treatment.

These might be expected to be particularly associated with the most potent of inhibitors and to be greater than with tamoxifen which has oestrogen agonistic properties. However, there may be differences between class of inhibitors and the steroidal agents may be less problematic if, because of their structure, they have androgenic properties on bone and other normal target tissues [57,58].

Clinical trials are needed to address these issues and, by the nature of the questions, these will require to be large and have an extensive follow up. Such trials are ongoing and are summarised below.

The only published randomised controlled trial of an AI as adjuvant therapy compares anastrozole with tamoxifen and is known as the Arimidex, Tamoxifen Alone or in Combination (ATAC) trial [59]. It recruited 9366 postmenopausal women with invasive breast cancer from 381 centres in 21 countries between July 1996 and March 2000. After completing primary therapy (surgery +/- radiotherapy +/- chemotherapy) patients were randomised to receive anastrozole plus placebo, tamoxifen plus placebo or anastrozole and tamoxifen together for 5 years as adjuvant therapy. The groups had similar demographics, tumour characteristics and primary treatment. Approximately 83% of tumours in each group were ER-positive and 7% ER negative, with the remainder unknown. Preliminary results of the trial were reported when median duration of therapy was 33.3 months [59].

The results showed anastrozole to be superior to tamoxifen in terms of disease-free survival in both the overall population (hazard ratio 0.83 [95% confidence interval (CI) 0.71 – 0.96], p = 0.013) and the ER-positive subgroup. There were also significantly fewer new contralateral primary tumours in the anastrozole-treated group (odds ratio 0.42 [0.22 – 0.79], p = 0.007). Interestingly, the combined arm did not show any significant improvement over tamoxifen alone. Although pharmacokinetics changes may explain the reduced efficacy of the combination, the suppression of oestrogen concentrations was similar in the two groups [60]. It is more likely that the differing effects of the agents on the ER account for the combined arm having no better efficacy than tamoxifen alone. In postmeno-

Table 7. Primary and secondary efficacy end point results of trial P024 comparing 4 months of neoadjuvant letrozole versus tamoxifen, in all study patients [54].

Efficacy end points	Letrozole (n = 154) %	Tamoxifen (n = 170) %	p Value
Primary end point			
Clinical response (palpation)	55	36	< 0.001
- Complete	10	4	
- Partial	45	32	
Secondary end points			
Ultrasound response	35	25	0.042
- Complete	3	1	
- Partial	32	24	
Mammographic response	34	16	< 0.001
- Complete	4	0	
- Partial	30	16	
Breast-conserving surgery	45	35	0.022

pausal women the effect of tamoxifen is to saturate the ERs and therefore act predominantly as an oestrogen antagonist. However, it has well-documented partial agonist effects. In contrast, anastrozole has no agonist effects, causing serum oestradiol levels to become extremely low and obliterating oestrogenic signalling through the receptor. When the two agents are combined, tamoxifen may be more likely to have oestrogenic activity because of the lower level of oestrogen resulting from aromatase inhibition by anastrozole. Thus anastrozole decreases the oestradiol levels with the same potency, but it has no effect in stopping tamoxifen binding to the ERs and inducing its partial agonist effects. This is the most likely explanation for the combined arm having similar results to the tamoxifen alone arm [59]. Recurrence and survival data from ATAC was updated and presented in San Antonio in December 2002. The efficacy advantages of anastrozole over tamoxifen were maintained with a 92.2 versus 89.6% 4-year recurrence-free rate in receptor-positive patients aged over 50 years.

Whilst the ATAC trial is the only investigation to have published results, there are other potentially important trials in progress. These are summarised in **Figure 5**. These vary in terms of design but have been particularly interested in the use of AIs in sequence with tamoxifen in adjuvant postmenopausal breast cancer. Two main trial models have been established, one giving an AI after 5 years of tamoxifen treatment or alternatively comparing tamoxifen and AIs alone or in sequence during the first 5 postoperative years.

These trials will also provide important data regarding the longer term toxicities of AIs. Results to date suggest that, in direct comparisons with tamoxifen, AIs appear to have a better toxicity profile. Fewer patients have to stop therapy because of drug-related side effects [52]. They have a reduced rate of thromboembolic events compared with tamoxifen. In addition, the ATAC data suggests that the rate of endometrial cancer may be reduced to the normal age-matched incidence [59]. This, together with the reduced rate of vaginal bleeding on AI, could have important economic implications because the current costs of thoroughly investigating women on tamoxifen who complain of postmenopausal bleeding are expensive.

The ATAC study also demonstrated a lower rate of hot flushes, vaginal dryness, endometrial cancer, cerebrovascular events, venous thromboembolism and deep venous thrombosis (DVT) with anastrozole compared with tamoxifen. There was an increased rate of musculoskeletal disorders and fractures with anastrozole. Musculoskeletal problems appear to be more common with anastrozole than letrozole based on a study directly comparing the tolerability of these two drugs. The increase in fracture rate relates to loss of bone density associated with the decrease in oestrogen levels and it is likely to also be a problem with letrozole. Exemestane has androgenic effects and could be associated with a lower rate of bone problems, but as yet there are no clinical data to confirm this. Ongoing studies are evaluating whether bisphosphonates given synchronously to women at highest risk of fracture can reduce the rate of fracture development. The effect of AIs on lipids is unclear. A small study of patients treated with letrozole indicated that it had adverse effects on lipid profile [61]. The only data for anastrozole are in the metastatic setting and show no major change in lipids [62].

AIs may currently be considered as first-line therapy in patients at high risk of DVT and pulmonary embolism [52]. They may also be recommended as first-line adjuvant therapy in elderly patients who have a higher rate of adverse effects on tamoxifen, where quality of life issues may be more pertinent than survival from breast cancer [52].

8. Hormone prevention of breast cancer

Risk to breast cancer has a strong hormonal aetiology and overexposure to oestrogen is thought to promote the disease [63]. Conversely, endocrine deprivation may prevent breast cancer [64] and a recent trial of tamoxifen as a preventative agent has produced promising results [65]. Hence, it may be that more efficacious endocrine-depriving agents can produce more beneficial effects. In this respect, the different mechanisms of action of antioestrogens and AIs may be important.

Most notably, tamoxifen blocks the action of oestrogen at the level of the ER, which results in blockade of receptor-mediated signals including proliferation in hormone-dependent breast cancers. However, tamoxifen (unlike AIs) does not reduce oestrogen levels. This may be crucial because there is a suggestion that metabolites of oestrogen are carcinogenic [66]; AIs which reduce oestrogens may, therefore, be doubly effective in both affecting initiation and promotion of cancer. Against this, AIs have not been used successfully in premenopausal women, a large population who may be candidates for hormone prevention. Trials are therefore underway to investigate the potential of AIs as preventative agents.

Additionally, ongoing adjuvant trials (**Figure 5**) will provide information about the occurrence of new contralateral breast cancers and side-effect profiles.

Pilot studies of chemoprevention using third generation AIs are being planned, as reviewed by Goss [67]. They largely involve women at high risk of breast cancer. The National Institute of Canada is conducting a pilot double-blind, multicentre trial to evaluate the effect of letrozole or placebo on breast density of postmenopausal women with high breast density. After the International Breast Cancer Intervention Study (IBIS) showed that tamoxifen significantly decreased the risk of developing breast cancer in high risk women when compared with placebo, there are plans to compare anastrozole with placebo in IBIS II. In another chemoprevention trial in progress, an AI is given to women with atypical or epithelial hyperplasia assessing reduction of hyperplasia or progression. Biomarkers of breast cancer risk are being used as end points in postmenopausal women with ductal carcinoma *in situ* (DCIS) and/or low risk invasive cancer [67].

It has been speculated that low doses of very potent AIs, for example, letrozole, may be able to block *in situ* oestrogen synthesis in the breast without interfering with ovarian oes-

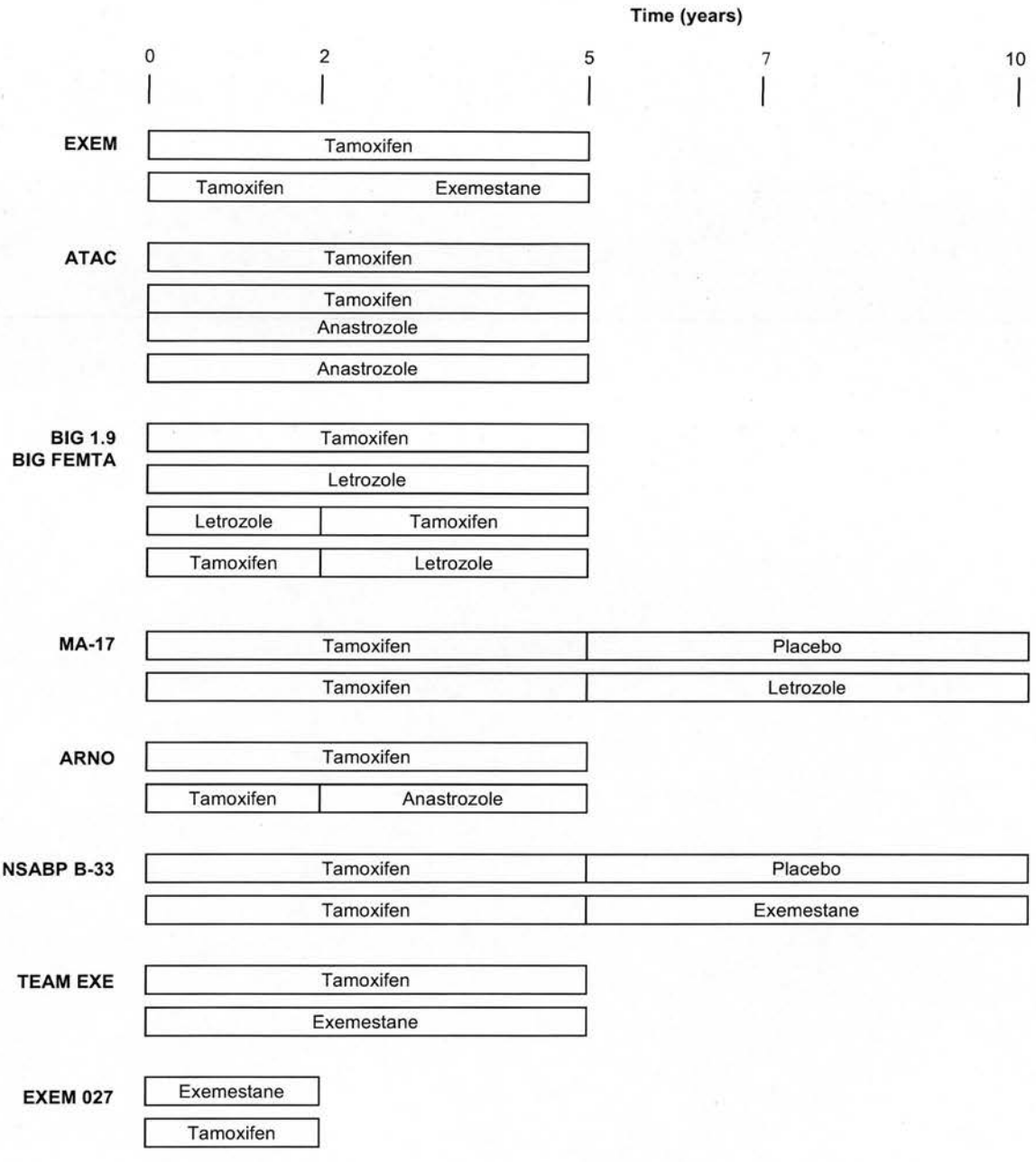

Figure 5. Ongoing trials using tamoxifen and aromatase inhibitors as adjuvant therapy in early breast cancer.

trogen production in premenopausal women [68]. This would potentially allow the agents to be used in chemoprevention without having adverse effects on other oestrogen-dependent tissues.

9. Other indications for aromatase inhibitors

Recently AIs have exclusively been used in postmenopausal women. This is because experience with prototype drugs indicate inadequate endocrine suppression and poor clinical benefit [69]. The problem is that in premenopausal women, levels of aromatase in the ovary are high and difficult to block; even if this is achieved, the resultant fall in circulating oestrogens results in decreased feedback inhibition at the hypothalamus and pituitary so that levels of gonadotrophins rise resulting in secondary increases both in androgen substrate and aromatase in the ovary. It may be that the later generation of highly potent AIs have sufficient power to overcome these secondary effects and use in premenopausal women may be a future option. However, caution may need

to be exerted as tropic effects on functioning ovaries may result in unforeseen pathology. Combining AIs with luteinising hormone-releasing hormone (LHRH) analogues may overcome these problems.

Should use in premenopausal women become viable, it opens the possibility of using AIs to treat benign breast conditions such as cyclic breast pain, fibroadenomata and recurrent cystic disease, which occur in women before the menopause.

There are also a variety of hormone-dependent conditions such as gynaecomastia [70], uterine fibroids/neoplasia [71], ovarian cancer [72] and prostate cancers [73], for which endocrine therapy might prove beneficial.

Aromatase has been shown to be of importance in the aetiology of gynaecomastia, with *in vitro* studies showing increased aromatase activity in skin fibroblasts from patients with gynaecomastia when compared with controls [74]. In 1986, Zachmann and colleagues published a small trial giving testolactone (150 mg t.i.d. for 2 – 6 months) to 22 boys with pubertal gynaecomastia [75]. Zachmann showed a gradual reduction in breast size over the treatment period with few unfavourable side effects. It is therefore surprising that no studies have been published looking at the effects of third generation AIs in this group. An advantage of these drugs is that they could be given by a once-daily oral dose. However, concerns have been raised about the effects on testicular function after animal models showed Leydig cell hypertrophy and hyperplasia and disturbed spermatogenesis [76]. It therefore remains to be established whether the potential benefits outweigh any side effects.

Although androgen deprivation therapy remains the mainstay of treatment for prostatic cancer, preclinical studies have suggested that oestrogens may have an important role in the development and progression of prostatic cancer [75]. Initial studies using the antioestrogen tamoxifen in metastatic prostate cancer produced disappointing results with response rates of between 0 and 23% reported [75]. In contrast, a small Phase I/II study using the first generation AI rogletimide gave more promising results [77]. Studies are being performed to assess the effects of the newer third generation AIs on prostate cancer.

It has been established that aromatase is expressed in endometriotic tissue in contrast to being undetectable in normal endometrial tissue [73]. The presence of aromatase results in local oestrogen and consequently prostaglandin E_2 (PGE_2; a potent inducer of aromatase activity in endometriotic stromal cells) production. This results in a positive feedback loop. It may, therefore, theoretically be possible to treat endometriosis with AIs. One case where this proved successful was described by Bulun and colleagues [78]. In this case, the biopsied endometriotic tissue showed abnormally high levels of aromatase mRNA, which might account for the marked success of therapy. Further studies need to be performed to determine the role of AIs in treating endometriosis, resistant to standard regimes.

Although AIs have been used occasionally in the past, it has usually been with early generation inhibitors and poor results. The new generation inhibitors offer new opportunities and the promise of greater success.

10. Expert opinion

The latest generation of AIs, as exemplified by anastrozole, exemestane and letrozole, are extremely specific and potent endocrine agents. In postmenopausal women, they reduce circulating oestrogens to levels not previously seen with other drugs. It might, therefore, be expected that when used against breast cancers that require hormones for growth they will produce profound antitumour effects. Clinical trials in advanced disease have now shown that this is the case. All such studies have proven at least equivalence versus established endocrine agents and the evidence is gradually accumulating of superiority over the gold standard hormone treatment, tamoxifen. Advanced disease with high tumour burden, increased likelihood of resistance and difficulties in accurately assessing response is not necessarily the best setting to demonstrate increased efficacy. Results from neoadjuvant studies in which effects are monitored on primary tumours are therefore particularly interesting; these show clear superiority over tamoxifen in terms of response rates and decreased need for major surgery in women presenting with large primary cancers. These data have led to the inception of large randomised trials for adjuvant use. Preliminary results for the ATAC trial are highly promising and have led to the expectation that aromatase inhibition may replace tamoxifen as first-line adjuvant hormone therapy. However, some degree of caution is warranted; follow up is short and the effects of long-term use on normal tissues whose function is dependent upon oestrogen needs to be assessed fully. The reduction in contralateral cancers in women given adjuvant therapy raises the possibility of use as a chemopreventative. In this setting, AIs may have advantages over selective oestrogen receptor modulator (SERMs), which may abrogate the proliferation effects of oestrogen without blocking genotoxic effects of oestrogen metabolites. AIs which reduce oestrogen levels would be expected to do both and be more efficient chemoprevention agents in postmenopausal women. (It is likely that use in premenopausal women will require combination with an agent to block feedback responses in the pituitary.) Finally, the therapeutic potential of AIs stretches beyond postmenopausal breast cancer. There are a variety of benign and malignant diseases which appear dependent upon oestrogen and might be usefully targeted by aromatase inhibition.

Bibliography

Papers of special note have been highlighted as either of interest (•) or of considerable interest (••) to readers.

1. MILLER WR: Estrogens and breast cancer. In: *Estrogen and Breast Cancer.* RG Landes Company, Austen,TX, USA (1996):1-24.
2. MILLER WR, LANGDON SP: Steroid hormones and cancer: (III) observations from human subjects. *Eur. J. Surg. Oncol.* (1977) **23**(2):163-183.
3. WELLS SA Jr, SANTEN RJ, LIPTON A *et al.*:. Medical adrenalectomy with aminoglutethimide: clinical studies in postmenopausal patients with metastatic breast carcinoma. *Ann. Surg.* (1978) **187**(5):475-484.
4. SANTEN RJ, SAMOJLIK E, WORGUL TJ: Aminoglutethimide – product profile. In: *A Comprehensive Guide to the Therapeutic Use of Aminoglutethimide.* RJ Santen, IC Henderson (Eds), Karger, Basel, Switzerland (1981):101-160.
5. JOHANNESSEN DC, ENGAN T, DI SALLE E *et al.*: Endocrine and clinical effects of exemestane (PNU 155971), a novel steroidal aromatase inhibitor, in postmenopausal breast cancer patients: a Phase I study. *Clin. Cancer Res.* (1997) **3**(7):1101-1108.
6. JOHNSTON JO, METCALF BW: Aromatase: a target enzyme. In: *Novel Approaches to Cancer Chemotherapy,* London Academic Press (1984):307-328.
7. DIXON JM: Exemestane: a potent irreversible aromatase inactivator and a promising advance in breast cancer treatment. *Expert Rev. Anti-Cancer Ther.* (2002) **2**(3):267-275.
8. SANTEN RJ, MISBIN RI: Aminoglutethimide: review of pharmacology and clinical use. *Pharmacotherapy* (1981) **1**(2):95-120.
9. VAN DEN BOSSCHE HV, MOEREELS H, KOYMANS LM: Aromatase inhibitors-mechanisms for non-steroidal inhibitors. *Breast Cancer Res. Treat.* (1994) **30**(1):43-55.
10. BARONE RM, SHAMONKI IM, SIITERI PK, JUDD HL: Inhibition of peripheral aromatization of androstenedione to estrone in postmenopausal women with breast cancer using delta 1-testololactone. *J. Clin. Endocrinol. Metab.* (1979) **49**(5):672-676.
11. SEGALOFF A: Δ^1-Testololactone: clinical trials. *Can. Res.* (1982) **Suppl. 42**:3387-3388.
12. GRIFFITHS CT, HALL TC, SABA Z, BARLOW JJ, NEVINNY HB: Preliminary trial of aminoglutethimide in breast cancer. *Cancer* (1973) **32**:31-37.
13. CUNNINGHAM D, POWLES TJ, DOWSETT M *et al.*: Oral 4-hydroxyandrostenedione, a new endocrine treatment for disseminated breast cancer. *Cancer Chemother. Pharmacol.* (1987) **20**(3):253-255.
14. STEIN RC, DOWSETT M, COOMBES RC: Studies of formestane alone and in combination with goserelin in premenopausal breast cancer. *Adv. Clin. Oncol.* (1994) **2**:149-154.
15. MILLER WR: Biology of aromatase inhibitors: pharmacology/endocrinology within the breast. *Endocr. Relat. Cancer* (1999) **6**(2):187-195.
16. DOWSETT M: Drug and hormone interactions of aromatase inhibitors. *Endocr. Relat. Cancer* (1999) **6**(2):181-185.
17. MILLER WR, MULLEN P, SOURDAINE P, WATSON C, DIXON JM, TELFORD J: Regulation of aromatase activity within the breast. *J. Steroid Biochem. Mol. Biol.* (1997) **61**(3-6):193-202.
18. BHATNAGAR A, MILLER WR: Pharmacology of inhibitors or estrogen biosynthesis. In: *Estrogens and Anti-Estrogens II: Pharmacology and Clinical Applications of Estrogens and Anti-Estrogens.* Oertel M, Schillinger E (Eds), Springer-Verlag, Berlin, Germany (1999):223-230.
19. KAO YC, OKUBO T, SUN XZ, CHEN S: Induction of aromatase expression by aminoglutethimide, an aromatase inhibitor that is used to treat breast cancer in postmenopausal women. *Anti-Cancer Res.* (1999) **19**(3A):2049-2056.
20. CHEN S, ZHOU D, OKUBO T, KAO YC, YANG C: Breast tumor aromatase: functional role and transcriptional regulation. *Endocr. Relat. Cancer* (1999) **6**(2):149-156.
21. HARADA N, HONDA SI, HATANO O: Aromatase inhibitors and enzyme stability. *Endocr. Relat. Cancer* (1999) **6**(2):211-218.
22. MILLER WR, O'NEILL J: The importance of local synthesis of estrogen within the breast. *Steroids* (1987) **50**(4-6):537-548.
23. MILLER WR, VIDYA R, MULLEN P, DIXON JM: Induction and suppression of aromatase by inhibitors. In: *Aromatase Inhibition and Breast Cancer.* WR Miller and RJ Santen (Eds), Marcel Dekker Inc., New York (2001):213-225.
24. LONNING PE: Pharmacology of new aromatase inhibitors. *Breast* (2001) 5:202-208.
25. DOWSETT M: Aromatase inhibition: the outcome of 20 years of drug development. In: *Aromatase Inhibition and Breast Cancer.* WR Miller, RJ Santen (Eds), Marcel Dekker Inc., New York (2001):3-15.
26. YATES RA, DOWSETT M, FISHER GV, SELEN A, WYLD PJ: Arimidex (ZD1033): a selective, potent inhibitor of aromatase in postmenopausal female volunteers. *Br. J. Cancer* (1996) **73**(4):543-548.
27. DEMERS LM, LIPTON A, HARVEY HA *et al.*: The efficacy of CGS 20267 in suppressing estrogen biosynthesis in patients with advanced stage breast cancer. *J. Steroid Biochem. Mol. Biol.* (1993) **44**(4-6):687-691.
28. THURLIMANN B, PARIDAENS R, SERIN D *et al.*: Third-line hormonal treatment with exemestane in postmenopausal patients with advanced breast cancer progressing on aminoglutethimide: a Phase II multicentre multinational study. Exemestane Study Group. *Eur. J. Cancer* (1997) **33**(11):1767-1773.
29. IVESON TJ, SMITH IE, AHERN J, SMITHERS DA, TRUNET PF, DOWSETT M: Phase I study of the oral nonsteroidal aromatase inhibitor CGS 20267 in postmenopausal patients with advanced breast cancer. *Cancer Res.* (1993) **15**;53(2):266-270.
30. LONNING PE, KVINNSLAND S: Mechanisms of action of aminoglutethimide as endocrine therapy of breast cancer. *Drugs* (1988) **35**(6):685-710.
31. GROOM GV, GRIFFITHS K: Effect of the anti-oestrogen tamoxifen on plasma levels of lutenizing hormone, follicle stimulating hormone, prolactin, oestradiol and progesterone in normal premenopausal women. *J. Endocrinol.* (1976) **70**:421-428.
32. O'REGAN RM, JORDAN VC: The evolution of tamoxifen therapy in breast cancer: selective oestrogen-receptor modulators and downregulators. *Lancet Oncol.* (2002) **3**(4):207-214.
33. MILLER WR, DIXON JM, MACFARLANE L, CAMERON D, ANDERSON TJ: Pathological features of breast cancer response following

neoadjuvant treatment with either letrozole or tamoxifen. *Eur. J. Cancer* (2002) (In press).

34. LIPTON A, DEMERS LM, HARVEY HA *et al.*: Letrozole (CGS 20267). A Phase I study of a new potent oral aromatase inhibitor of breast cancer. *Cancer* (1995) **15**;75(8):2132-2138.

35. JOHNSTON SR, HAYNES BP, SACKS NP *et al.*: Effect of oestrogen receptor status and time on the intra-tumoural accumulation of tamoxifen and N-desmethyltamoxifen following short-term therapy in human primary breast cancer. *Breast Cancer Res. Treat.* (1993) **28**(3):241-250.

36. JONAT W, HOWELL A, BLOMQVIST C *et al.*: A randomised trial comparing two doses of the new selective aromatase inhibitor anastrozole (Arimidex) with megestrol acetate in postmenopausal patients with advanced breast cancer. *Eur. J. Cancer* (1996) **32A**(3):404-412.

37. BUZDAR A, JONAT W, HOWELL A *et al.*: Anastrozole, a potent and selective aromatase inhibitor, versus megestrol acetate in postmenopausal women with advanced breast cancer: results of overview analysis of two Phase III trials. Arimidex Study Group. *J. Clin. Oncol.* (1996) **14**(7):2000-2011.

- **Large randomised trial comparing anastrozole and megestrol acetate in advanced breast cancer.**

38. GERSHANOVICH M, CHAUDRI HA, CAMPOS D *et al.*: Letrozole, a new oral aromatase inhibitor: randomised trial comparing 2.5 mg daily, 0.5 mg daily and aminoglutethimide in postmenopausal women with advanced breast cancer. Letrozole International Trial Group (AR/BC3). *Ann. Oncol.* (1998) **9**(6):639-645.

- **Large randomised trial comparing letrozole and aminoglutethimide in advances breast cancer.**

39. DOMBERNOWSKY P, SMITH I, FALKSON G *et al.*: Letrozole, a new oral aromatase inhibitor for advanced breast cancer: double-blind randomized trial showing a dose effect and improved efficacy and tolerability compared with megestrol acetate. *J. Clin. Oncol.* (1998) **16**(2):453-461.

- **Large randomised trial comparing letrozole and megestrol acetate in advanced breast cancer.**

40. KAUFMANN M, BAJETTA E, DIRIX LY *et al.*: Exemestane is superior to megestrol acetate after tamoxifen failure in postmenopausal women with advanced breast cancer: results of a Phase III randomized double-blind trial. The Exemestane Study Group. *J. Clin. Oncol.* (2000) **18**(7):1399-1411.

- **Large randomised trial comparing exemestane and megestrol acetate in advanced breast cancer.**

41. BUZDAR A, DOUMA J, DAVIDSON N *et al.*: Phase III, multicenter, double-blind, randomized study of letrozole, an aromatase inhibitor, for advanced breast cancer versus megestrol acetate. *J. Clin. Oncol.* (2001) **19**(14):3357-3366.

- **Large randomised trial comparing letrozole and megestrol acetate in advanced breast cancer.**

42. ROSE C, VTORAYA O, PLUZANSKA A *et al.*: Letrozole (Femara) versus anastrozole (Arimidex): second-line treatment in postmenopausal women with advanced breast cancer. *38th Annual Meeting of the American Society of Clinical Oncology*, Orlando, FL, USA (2002) (Abstract 131).

43. JONES S, CHANG A, LUSCH C *et al.*: A Phase II confirmatory study of antitumor efficacy and safety of exemestane (EXE) as third-line hormonal treatment of postmenopausal patients (pts) with metastatic breast cancer (MBC) refractory to tamoxifen and Megace™. *Breast Cancer Res. Treat.* (1998) **50**:305 (Abstract 437).

44. JONES S, VOGEL C, ARKHIPOV A *et al.*: Multicenter, Phase II trial of exemestane as third-line hormonal therapy of postmenopausal women with metastatic breast cancer. Aromasin Study Group. *J. Clin. Oncol.* (1999) **17**:3418-3425.

45. LONNING PE, BAJETTA E, MURRAY R *et al.*: Activity of exemestane in metastatic breast cancer after failure of nonsteroidal aromatase inhibitors: a Phase II trial. *J. Clin. Oncol.* (2000) **18**:2234-2244.

46. BUZDAR AU, JONAT W, HOWELL A *et al.*: Anastrozole versus megestrol acetate in the treatment of postmenopausal women with advanced breast carcinoma: results of a survival update based on a combined analysis of data from two mature Phase III trials. Arimidex Study Group. *Cancer* (1998) **83**(6):1142-1152.

47. MOURIDSEN H, GERSHANOVICH M, SUN Y *et al.*: Superior efficacy of letrozole versus tamoxifen as first-line therapy for postmenopausal women with advanced breast cancer: results of a Phase III study of the International Letrozole Breast Cancer Group. *J. Clin. Oncol.* (2001) **19**(10):2596-2606.

48. MOURIDSEN H, SUN Y, GERSHANOVICH M *et al.*: Final survival analysis of the double-blind, randomized, multinational Phase III trial of letrozole (Femara®) compared to tamoxifen as first-line hormonal therapy for advanced breast cancer. *Breast Cancer Res. Treat.* (2001) **69**:211.

49. NABHOLTZ JM, BUZDAR A, POLLAK M *et al.*: Anastrozole is superior to tamoxifen as first-line therapy for advanced breast cancer in postmenopausal women: results of a North American multicenter randomized trial. Arimidex Study Group. *J. Clin. Oncol.* (2000) **18**(22):3758-3767.

- **Large randomised trial comparing anastrozole and tamoxifen as first-line therapy for advanced breast cancer.**

50. BONNETERRE J, THURLIMANN B, ROBERTSON JF *et al.*: Anastrozole versus tamoxifen as first-line therapy for advanced breast cancer in 668 postmenopausal women: results of the Tamoxifen or Arimidex Randomized Group Efficacy and Tolerability study. *J. Clin. Oncol.* (2000) **18**(22):3748-3757.

- **Large randomised trial comparing anastrozole and tamoxifen as first-line therapy for advanced breast cancer.**

51. DIXON JM, LOVE CD, BELLAMY CO *et al.*: Letrozole as primary medical therapy for locally advanced and large operable breast cancer. *Breast Cancer Res. Treat.* (2001) **66**(3):191-199.

52. GOSS PE, STRASSER K: Aromatase inhibitors in the treatment and prevention of breast cancer. *J. Clin. Oncol.* (2001) **19**(3):881-894.

- •• **Excellent review of the current and potential uses of AIs.**

53. DIXON JM, RENSHAW L, BELLAMY C, STUART M, HOCTIN-BOES G, MILLER WR: The effects of neoadjuvant anastrozole (Arimidex) on tumor volume in postmenopausal women with breast cancer: a randomized, double-blind, single-center study. *Clin. Cancer Res.* (2000) **6**(6):2229-2235.

54. DIXON JM, ANDERSON TJ, MILLER WR: Neoadjuvant endocrine therapy of breast cancer: a surgical perspective. *Eur. J. Cancer* (2002) **38**(17):2214-2221.

- **Discussion of the use of AIs in the neoadjuvant setting.**

55. ANDERSON TJ, DIXON JM, STUART M, SAHMOUD T, MILLER WR: Effect of

neoadjuvant treatment with anastrozole on tumour histology in postmenopausal women with large operable breast cancer. *Br. J. Cancer* (2002) **87**:334-338.

56. EIERMANN W, PAEPKE S, APPFELSTAEDT J: Letrozole Neo-Adjuvant Breast Cancer Study Group. Preoperative treatment of postmenopausal breast cancer patients with letrozole: A randomized double-blind multicenter study. *Ann. Oncol.* (2001) **12**(11):1527-1532.

57. GOSS P: Anti-aromatase agents in the treatment and prevention of breast cancer. *Cancer Control* (2002) **9**(2, Suppl.):2-8.

• **Discussion about uses of AIs in the treatment and prevention of breast cancer.**

58. GOSS P: Chemoprevention with aromatase inhibitors. In: *Aromatase Inhibition and Breast Cancer.* WR Miller, RJ Santen (Eds), Marcel Dekker Inc., New York (2001):161-181.

59. THE ATAC TRIALISTS' GROUP: Anastrozole alone or in combination with tamoxifen versus tamoxifen alone for adjuvant treatment of postmenopausal women with early breast cancer: first results of the ATAC randomised trial. *Lancet* (2002) **359**(9324):2131-2139.

•• **First large double-blind randomised trial of anastrozole versus tamoxifen versus combination of anastrozole and tamoxifen as adjuvant treatment for early breast cancer.**

60. THE ATAC TRIALISTS' GROUP: Pharmacokinetics of anastrozole and tamoxifen alone, and in combination, during adjuvant endocrine therapy for early breast cancer in postmenopausal women: a sub-group of the 'Arimidex' and Tamoxifen Alone or in Combination (ATAC) trial. *Br. J. Cancer* (2001) **85**:317-324.

61. ELISAF MS, BAIRAKTARI ET, NICOLAIDES C *et al.*: Effect of letrozole on the lipid profile in postmenopausal women with breast cancer. *Eur. J. Cancer* (2001) **37**:1510-1513.

62. DEWAR J, NABHOLTZ JM, BONNETERRE J, BUZDAR AU: The effect of anastrozole (Arimidex) on serum lipids: a randomised comparison of anastrozole (AN) versus tamoxifen (TAM) in postmenopausal women with advanced breast cancer. *Eur. J. Cancer* (2001) **37**(Suppl. 5):5.

63. THOMAS HV, KEY TJ, ALLEN DS *et al.*: A prospective study of endogenous serum hormone concentrations and breast cancer risk in post-menopausal women on the island of Guernsey. *Br. J. Cancer* (1997) **76**(3):401-405.

64. MILLER WR: Estrogens and endocrine therapy for breast cancer. In: *Estrogen and Breast Cancer.* RG Landes Company, Austen, TX, USA (1996):125-150.

65. FISHER B, COSTANTINO JP, WICKERHAM DL *et al.*: Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. *J. Natl. Cancer Inst.* (1998) **90**(18):1371-1388.

66. YAGER JD, LIEHR JG: Molecular mechanisms of estrogen carcinogenesis. *Ann. Rev. Pharmacol. Toxicol.* (1996) **36**:203-232.

67. GOSS PE: Aromatase inhibitors and chemoprevention of breast cancer. In: *Endocrine Therapy in Breast Cancer.* WR Miller, JN Ingle (Eds). Marcel Dekker, Basel, CH (2002):309-317.

68. SANTEN RJ, YUE W, NAFTOLIN F, MOR G, BERSTEIN L: The potential of aromatase inhibitors in breast cancer prevention. *Endocr. Relat. Cancer* (1999) **6**(2):235-243.

69. HARRIS AL, DOWSETT M, JEFFCOATE SL, MCKINNA JA, MORGAN M, SMITH IE: Endocrine and therapeutic effects of aminoglutethimide in premenopausal patients with breast cancer. *J. Clin. Endocrinol. Metab.* (1982) **55**(4):718-722.

70. KAPLOWITZ PB: Aromatase inhibitors as therapy for pubertal gynecomastia. In: *Aromatase Inhibition and Breast Cancer.* WR Miller, RJ Santen (Eds), Marcel Dekker Inc., New York (2001):259-266.

71. BULUN SE, ZEITON KM, TAKAYAMA K, SASANO H, SIMPSON ER: Aromatase in endometriosis: biological and clinical application. In: *Aromatase Inhibition and Breast Cancer.* WR Miller, RJ Santen (Eds), Marcel Dekker Inc., New York (2001):279-298.

72. BOWMAN A, GABRA H, LANGDON SP *et al.*: CA125 Response is associated with estrogen receptor expression in a Phase II Trial of letrozole in ovarian cancer: identification of an endocrine-sensitive subgroup. *Clin. Cancer Res.* (2002) **8**(7):2233-2239.

73. SMITH MR: Aromatase inhibition and prostate cancer. In: *Aromatase Inhibition and Breast Cancer.* WR Miller, RJ Santen (Eds), Marcel Dekker Inc., New York (2001):271-278.

74. BULARD J, MOWSZOWICZ I, SCHAISON G: Increased aromatase activity in pubic skin fibroblasts from patients with isolated gynecomastia. *J. Clin. Endocrinol. Metab.* (1987) **64**(3):618-623.

75. ZACHMANN M, EIHOLZER U, MURITANO M, WERDER EA, MANELLA B: Treatment of pubertal gynaecomastia with testolactone. *Acta Endocrinol. Suppl. (Copenh.)* (1986) **279**:218-226.

76. JUNKER WALKER U, NOGUES V: Changes induced by treatment with aromatase inhibitors in testicular Leydig cells of rats and dogs. *Exp. Toxicol. Pathol.* (1994) **46**(3):211-213.

77. DEARNALEY DP, SHERARER RJ, GALLACHER C *et al.*: A Phase (Ph) I/II study with rogletimide (ROG) in patients (pt) with hormone resisitant (HR) prostatic cancer (CaP) (meeting abstr). *Ann. Oncol.* (1995) (Suppl. 8):179.

78. TAKAYAMA K, ZEITOUN K, GUNBY RT, SASANO H, CARR BR, BULUN SE: Treatment of severe postmenopausal endometriosis with an aromatase inhibitor. *Fertil. Steril.* (1998) **69**(4):709-713.

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Published Abstracts

Short- and Long-Term Effects of Letrozole on Tumor Histopathology and Immunopathology in Patients with Breast Cancer given Neoadjuvant Treatment. **Jackson J**, White S, Dixon JM, Anderson TJ, Renshaw L, Miller WR. . Breast Cancer Research and Treatment vol 77, supp 1, Dec 2003

Neoadjuvant Letrozole : The Edinburgh Experience. **Jackson J**, Dixon JM, Cameron DA, Miller WR European Journal of Cancer. Sep 2003 vol 37 supp 5

Is there an optimal duration of neoadjuvant letrozole therapy? Renshaw L, **Murray J**, Young O, Cameron DC, Miller WR, Dixon JM. Breast Cancer Reseacrh and Treatment vol 78 supp 1, Dec 2004

Microarray analysis of sequential tumour biopsies from patients receiving neoadjuvant therapy is able to distinguish sub-populations of breast cancers with differential response to the aromatase inhibitor, letrozole. Miller WR, Renshaw L, **Murray J**, Larionov A, Anderson TJ, White S, Hampton G, Walker JR, Ho S, Krause A, Evans DB, Dixon JM. Breast Cancer Reseacrh and Treatment vol 78 supp 1, Dec 2004

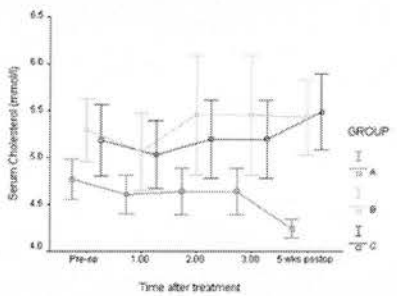
Early changes in tumour Ki67 expression differentiate for pathological (but not clinical) response in breast cancers treated neo-adjuvantly with letrozole. Anderson TJ, Dixon JM, **Murray J**, Renshaw L, White S, Miller WR. Breast Cancer Reseacrh and Treatment vol 78 supp 1, Dec 2004

Prediction of hormone response in breast cancer by microarray analysis of sequential tumour biopsies from patients receiving neoadjuvant therapy with letrozole. Miller WR, Renshaw L, **Murray J**, Larionov A, Anderson TJ, White S, Hampton G, Walker JR, Ho S, Krause A, Evans DB, Dixon JM. European Journal of Cancer. Sep 2005 vol 3 supp 1

Neoadjuvant letrozole is equally effective in Her 2 positive and negative breast cancers. Young O, **Murray J**, Renshaw L, Evans DB, Cameron D, Dowsett M, Miller WR, Dixon JM. European Journal of Cancer. Sep 2005 vol 3 supp 1

C). End point assessments include clinical and pathologic responses, changes in cholesterol and lipid profiles and tolerability. For those patients with clinical response, assigned treatment continued adjuvantly post-surgery.

Results: From Feb 2002 to April 2003, a total of 41 patients were recruited. Twenty-nine patients have completed 3 months of treatment and were operated. The tumor size shrunk to 3 cm or less for 65% of the patients, who were then suitable for conservative surgery. However, only one patient decided for lesser surgery. All except one of the patients tolerated the treatment well. One patient (group A) developed allergic skin rashes and withdrew from treatment. The clinical response rates were 61.5%, 60% and 54.5% respectively for Groups A, B and C. The cholesterol levels (figure 1) for group A patients dropped progressively and statistical difference was observed between 5th week after operation and preoperative level (P=0.026). No difference was observed for the other groups.



Data on COX expression, and aromatase expression are being analyzed and the correlation with clinical response will be reported.

Discussion: The treatment with celecoxib and exemestane is well tolerated. This combination regimen poses an advantage over the anti-aromatase therapies in reducing the serum cholesterol profiles of postmenopausal patients with breast cancer.

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Type 1 insulin-like growth factor receptor expression and activation in clinical breast cancer.

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Background: Insulin-like growth factors (IGFs) bind to and activate the type 1 insulin-like growth factor receptor (IGF-1R). IGF-1R signalling is a dominant pathway for endocrine responsive breast cancer *in vitro*, including MCF-7. In such cells, IGF-1R expression, activation and its signalling are prominent and IGFs are growth promoting. However, associations between IGF-1R expression, endocrine response and prognostic features are only poorly documented in clinical disease, and receptor activation has not to date been investigated *in vivo*.

Materials and Methods: In the present study, IGF-1R expression and activation has been immunocytochemically-assessed in an archival clinical breast cancer series (n=65). IGF-1Rα antibody and a phospho-specific IGF-1R antibody (detecting Y1316 phosphorylation in the kinase domain) were used to monitor receptor expression and activation respectively, employing peroxidase-labelled polymer detection. Antibody validity was confirmed in MCF-7 with/without IGF-1 treatment.

Results: IGF-1R expression and activation were heterogeneously detected in the tumour epithelial cells of most breast cancers. Immunostaining was principally at the plasma membrane and diffusely in the cytoplasm. Staining in these compartments positively associated ($p<0.001$). Plasma membrane IGF-1R expression directly associated with oestrogen receptor (ERα; $p=0.028$) and progesterone receptor ($p=0.041$), and indirectly with epidermal growth factor receptor (EGFR; $p=0.02$) and grade ($p=0.067$). Increased IGF-1R expression also associated with responsive disease at 6 months ($p=0.03$) and an extended time to progression on endocrine therapy ($p=0.009$). Associations were lost on ERα subdivision. Plasma membrane IGF-1R activation directly correlated with IGF-1R expression ($p<0.001$). Subdivision by IGF-1R expression and activation revealed response at 6 months ($p=0.049$) and increased time to progression on endocrine therapy ($p=0.0174$) when both expression and activation were elevated. Some association was also observed in ERα positive disease, although this proved non-significant. There were no associations with patient survival.

Discussion: IGF-1R is enriched in ERα positive/well-differentiated/EGFR negative and endocrine responsive clinical breast cancer. Endocrine responsive disease also has elevated IGF-1R activation (as in MCF-7) suggesting IGF-1R signalling is important to these tumours *in vivo*. The impact of endocrine treatments on IGF-1R signalling and the role of IGF-1R at relapse should now be examined *in vivo* using these new assays for receptor expression and activation.

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Short and long term effects of letrozole on tumour histo- and immuno-pathology in breast cancer patients given neoadjuvant treatment.

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Background: Letrozole is an effective endocrine treatment in postmenopausal women with ER-rich cancers. Neoadjuvant therapy combined with sequential biopsy allows accurate monitoring of tumour changes with treatment. The objective of this study is to monitor the effects of letrozole on tumour grading features, ER, PgR (a marker of oestrogenic activity) and Ki67 (a marker of proliferation).

Materials and Methods: 50 postmenopausal women with large primary breast cancers were treated with neoadjuvant letrozole 2.5mg daily for 3 months. Tumour samples were taken at diagnosis, after 10-14 days and after 3 months of treatment. ER, PgR and Ki67 were assessed by immunohistochemistry; these and grading features were scored as described by Miller et al [Euro J Cancer 39 (2003) 462-468].

Results: At initial biopsy all cancers were scored ER 5+2 or 5+3 (by Allred score); 45 of 50 (90%) were PgR positive (range 1+2 to 5+3); Ki67 scores ranged from 5-60%. Changes with treatment from initial biopsy at diagnosis are summarised in the table.

	10 - 14 Days			3 Months		
	Increase	No Change	Decrease	Increase	No Change	Decrease
ER	0	50	0	0	50	0
PgR	0	14	36*	1	9	40*
Ki67	1	1	48*	0	1	49*

*$p<0.00001$ compared with initial biopsy, by paired Wilcoxon rank test

ER: the score was unaffected by treatment with letrozole.

PgR: By 10 –14 days, 25 of the PgR positive cases reduced to 0, 11 other cases decreased by both proportion and intensity but not to 0. Fourteen cases were unchanged, including the five that were negative. By 3 months, a further 5 PgR positive patients dropped to 0 and one of the PgR negative cancers became focally positive.

Ki67: Scores had decreased to 1% or less in 23 cases after 10-14 days of treatment; 32 cases had a score of 1% or less at 3 months. Ki67 scores subsequently increased between 10-14 days and 3 months in 5 cases.

Histological grading: By 10-14 days, 8 cases showed an increase in gland features, 13 cases showed a decrease, and 3 an increase in nuclear grading. 8 cases showed a decrease in mitosis.

Conclusion: Letrozole has been shown to have marked anti-oestrogenic and anti-proliferative effects in tumours within 10-14 days. These changes precede any clinically apparent indication of response.

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Multi-center clinical trials of trilostane (modrenal) for advanced breast cancer in post-menopausal women.

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Background: Trilostane (Modrenal) has been shown recently to modulate binding of estrogen to both ERα and ERβ and block cell proliferation in breast cancer cells mediated through both ERE and AP1-dependent pathways. In multi-center, international clinical trials a total of 783 post-menopausal women with advanced, progressing

261 Pharmacokinetic (PK) study to evaluate the combination of exemestane (E) and tamoxifen (T) in the treatment of metastatic breast cancer: preliminary results.

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E is a new Type I, steroidal aromatase inactivator that irreversibly binds to the aromatase enzyme. T has antiestrogenic and estrogenic properties and is probably the best known hormonal therapy for patients with breast cancer. The antitumor activity of E, given alone or in combination with T, was investigated in rats by Zaccheo et al (J Steroid Biochem Mol Biol 1993). The combined treatment resulted in higher antitumor activity compared to either agent alone. The increase in efficacy with this combination led us to design a pilot study in which toxicity and PK could be evaluated. Response was considered a secondary endpoint. Patients were eligible if they were postmenopausal, had either measurable or evaluable disease, or if they had prior tamoxifen and/or aromatase inhibitors either in the adjuvant or metastatic setting. Patients were not eligible if they had prior history of thromboembolic events, were on HRT, or were taking over-the-counter estrogenic supplements. Patients were given E 25 mg daily for 2 weeks. After the second week, patients continued on E and started on T 20 mg daily. Blood samples for E levels, estradiol, estrone, and estrone sulfate were collected on day 14 of the 2-week single agent E period and approximately 4 weeks after starting the combination treatment. Eighteen patients were registered in the study but only 17 underwent treatment and PK sampling. One patient withdrew consent and discontinued treatment. Median age was 62 years (range, 46-84) and all patients had a performance status of 1. Sixteen patients had received prior hormonal therapy, most of which had received more than one prior hormonal agent . Thirteen patients had received prior tamoxifen, 6 had received aromatase inhibitors (AIs), 2 toremifene, 2 progestins, and 2 had received androgens. Of the patients who had received prior AIs, 2 had previously received exemestane. All patients had estrogen and/or progesterone receptor-positive tumors. We have seen 1PR, 1MR, 3SD, 7PD, and 4 are too early to evaluate. The most common toxicity observed include: grade 1 fatigue, grade 1 / 2 hot flashes, grade 1 nausea, grade 1 vaginal bleeding, and grade 2 bone pain. PK analysis is currently being performed. Updated information, including PK analysis and estrogen levels, will be provided at a later time. The results of this study will help decide the need for further evaluation of this combination.

262 Anastrozole therapy does not compromise lipid metabolism in breast cancer patients previously treated with tamoxifen.

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Background: Tamoxifen (TAM) exerts beneficial influence on lipid profile as a result of its estrogen-like properties. A number of new-generation aromatase inhibitors used sequentially to the initial adjuvant TAM are being currently investigaed in clinical trials, including anastrozole (ANS). There are concerns, however, that ANS, as a potent supressor of estrogen, may reverse beneficial effects of TAM on the serum lipids profile. The current study updates at prolonged observation our previous results on effects of ANS used in sequence to TAM in breast cancer patients.

Materials and Methods: Analysis included 43 postmenopausal breast cancer women, converted to ANS (1mg/d.) after 14-234 weeks (median: 67) of TAM (20mg/d.) treatment used for advanced disease (N=25) or in adjuvant setting (N=18). Concentrations of basic blood lipids and body mass index values (BMI) were measured before treatment, and at minimum 24 (median: 26, range: 24 - 33) and 60 (median: 63, range: 60 - 70) weeks of ANS administration afterwards.

Results: there was no statistically significant change over time in basic lipid parametres, that included total (TCH)- (p=0.23), LDL- (p=0.26), and HDL-cholesterol (p=0.37), triglycerides (p=0.32), the number of patients with TCH≥200mg/dl (p=0.55), the atherogenic risk ratios: TCH/HDL (p=0.45) and LDL/HDL-cholesterol (p=0.39) as well as in mean BMI values (p=0.54).

Conclusion: administration of ANS for ≥ 60 months in sequence to TAM does not affect lipid profile of breast cancer patients.

263 Anastrozole demonstrates clinical and biological effectiveness in erbB2 ER positive breast cancers.

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22 postmenopausal women with large operable or locally advanced with oestrogen receptor (ER) rich breast cancers were randomised to receive 1mg or 10mg of anastrozole for 3 months following which they had surgery. The age range of women was from 56 to 92 ER levels were Allred score 5 (1 patient), 6 (3 patients), 7 (10 patients), 8 (8 patients). Responders continued on anastrozole post surgery for 5 years. Median follow up: 44 months.

All patients had erbB2 assessed in their initial biopsy using the HerceptTest. Response was assessed by clinical examination (pre) and ultrasound according to standard criteria (CR/PR complete or partial response, SD stable disease). Proliferation before and after 3 months (post) was assessed by Ki67. Progesterone receptor (PgR) was assessed before and after treatment.

		Clinical		Ultrasound		Median Ki67		Fall in
ErbB2	No	CR/PR	SD	CR/PR	SD	Pre	Post	PgR
0/1+	16	15	1	10	6	23.5	5+	13/13*
3+	6	6	0	5	1	22.5	7.5–	3/4*

*5 patients PgR 0 on first biopsy, –p=0.017, +p<0.0001

Response did not differ in relation to erbB2 status All 0/1+ and all 3+ patients had a reduction in proliferation. In the 16 patients with 0/1+ erbB2 tumours there have been 3 events (1 death, 1 local recurrence, 1 lung metastasis). In the 6 erbB2 3+ patients there have been 2 events (1 local recurrence → lung metastasis and 1 liver metastasis) both patients are still alive. These are the first data demonstrating the clinical and biological effectiveness of anastrozole in erbB2 positive ER positive breast cancers.

264 Neoadjuvant letrozole: the Edinburgh experience.

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The randomised neoadjuvant trial of letrozole versus tamoxifen reported a clinical response rate of 55% (85/154) for patients randomised to letrozole and a relationship between response and ER level.

83 postmenopausal patients with large operable or locally advanced ER rich breast cancers have been treated with 3 months of letrozole and response assessed clinically and volume changes over the 3 month study assessed by clinical measurement and ultrasound - 65 patients responded - overall response rate 78%, a significantly better response rate than in the 024 study, p=0.0004. Response rate did not differ significantly between ER categories but percentage reduction in volume did (Table).

ER Score	No of	No of	%	Median % Reduction in Tumour Volume	
Allred	Pts	Responders	Response	Clin	USS
8	60	48	80	76+	67+
6 + 7	23	17	74	63	48

+p<0.05

Letrozole is confirmed as being a highly effective agent at producing tumour shrinkage in postmenopausal women with ER rich breast cancers.

Is there an optimal duration of neoadjuvant letrozole therapy?

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Background: Randomised studies of neoadjuvant aromatase inhibitors have treated patients for 3 - 4 months. The aim of this review was to assess whether tumours continue to respond to neoadjuvant letrozole for periods longer than 3-4 months.

Patients and Methods: 142 postmenopausal women with large operable or locally advanced ER rich (ER Allred score 6 or more) breast cancer were enrolled into a prospective audit assessing response to neoadjuvant letrozole 2.5mg per day. Clincal response was assessed at 3 months; non responders and patients whose tumours had become operable or had responded sufficiently to allow breast conserving surgery proceeded to surgery. The remaining 42 patients who were either unfit for surgery, refused surgery, had responded but still required mastectomy or were inoperable, continued letrozole for a further 3 months. 22 patients continued letrozole for a total of 12 months. Reductions in tumour volume over the first 3 months were compared with 3-6 and a period of between 6-12 months were calculated.

Results: Median % reduction in the tumour volumes from 0-3 months, 3-6 months and 6-12 months are shown in the table.

	Number of Patients	Median	95% CI
% reduction from 0 - 3 months	42	52	37-62
% reduction from 3 - 6 months	42	57	26-100
% reduction from 6 - 12 months	22	66	22-100

Tumours continued to reduce in volume during the 12 months study period.

Complete responses: At 3 months there were 4/42 (9.5%) complete responses, by 6 months there were 12/42 (29%) and by 12 months 8/22 (36%). One patient who was responding at 3 months had disease progression at 12 months.

Conclusion: Neoadjuvant letrozole produces ongoing tumour shrinkage in postmenopausal women over 12 months in large operable or locally advanced ER+ breast cancers. Patients whose tumours are responding to letrozole at 3 months can expect further reduction in tumour volume with continued treatment. There is no optimum duration for use of neoadjuvant letrozole; it can be used safely for up to 12 months.

Microarray analysis of sequential tumour biopsies from patients receiving neoadjuvant therapy is able to distinguish sub-populations of breast cancers with differential response to the aromatase inhibitor, letrozole.

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Introduction: Microarray analysis of sequential tumour biopsies taken during neoadjuvant therapy permits identification of genes whose changes in expression correlate with response and resistance to treatment. The present study has analysed the molecular changes in individual breast cancers which occur within 14 days of starting therapy with letrozole.

Materials and methods: 81 postmenopausal women with large operable or locally advanced breast cancers treated with a 3 months of neoadjuvant letrozole (2.5mg daily) had tissue sampled at diagnosis, 14 days and 3 months. Tumours were monitored and response was based on clinical, ultrasound and mammographic changes. RNA was extracted and doubly amplified before hybridization on Affymetrix chips (HG_U133A).

Results: Sufficient good quality RNA for microarray was obtained from all 3 tumour samples in 69 patients. Analysis is complete on 56 cases. Based on differing clustering techniques including either the 100 or 1% (223) genes showing the greatest change or the 1000 highest average expressed levels, it was possible to identify highly consistent

patterns of changes in expression level with treatment. Furthermore, tumours could be subdivided into groups showing distinct patterns of molecular changes. The genes involved included known markers of hormone sensitivity (trefoil factors, PgR, LIV-1), tumour progression (cyclin B2, CDC28, BRCA1 associated RING domain 1, antigen identified by monoclonal antibody Ki67) as well as novel candidate genes. Tumour from remaining 13 cases are currently being analysed before microarray changes are correlated to clinical and pathological response data.

Conclusions: Changes in tumour gene expression in biopsies taken before and after 14 days treatment with neoadjuvant letrozole may define of tumour cohorts with differing response to letrozole. This should permit early recognition of response/resistance to the drug and add to our understanding of its mode of action.

Early changes in tumour Ki67 expression differentiate for pathological (but not clinical) response in breast cancers treated neo-adjuvantly with letrozole.

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Background: We have previously shown that neo-adjuvant treatment with the aromatase inhibitor, letrozole, produce marked anti-oestrogenic and anti-proliferative effects in tumours within 10-14 days. The objective of this study was to determine whether these changes were related to clinical and pathological responses as assessed after 3 months of treatment

Materials and Methods: 63 postmenopausal women with large primary breast cancers were treated with neoadjuvant letrozole 2.5mg daily for 3 months. Tumour samples were taken at diagnosis, after 10-14 days and after 3 months of treatment.
Oestrogen receptor(ER),progesterone receptor(PgR), Ki67 and morphology were assessed as described by Miller et al [Eur J Cancer 39 (2003) 462-468].

Results: 49(78%) patients had a clinical response(>50% reduction in tumour volume at 3month by serial ultrasound) and 44(75%) of 59 assessable tumours displayed evidence of a pathological response (decreased cellularity/increased fibrosis). Pre-treatment scores for Ki67 were similar in responders(R) and non-responders(NR) whether assessed clinically or by tumour pathology. Treatment was associated with highly significant Ki67 decreases in all tumour sub-groups (all at least $p< 0.005$ by paired Wilcoxon rank test) at 14days. Values (mean%±SEM) for pre-treatment vs 14days were 14.04±1.13 vs 5.04±0.96 for clinical responders, 15.81±2.12 vs 7.08±2.15 for clinical non-responders, 13.95±1.07 vs 4.28±0.88 for pathology responders and 15.97±2.22 v 9.35± 2.22 for

pathology non-responders. However, levels of Ki67 at 14days into treatment were not significantly different in clinical responders and NR, but scores were significantly higher in tumours which subsequently did not change pathology at 3months compared with those showing a pathological response. Parallel measurements of ER and PgR failed to detect differences in these parameters between clinical/pathological responding and non-responding tumours although significant decreases in expression of PgR were observed at 14days in all patient groups.

Conclusion: Letrozole is capable of producing profound decreases in expression of Ki67 and PgR although these do not always translate into clinical response. However, the expression of Ki67 at 14days is significantly higher in tumours which subsequently fail to show morphological evidence of response. It remains to determine whether these changes are associated with differences in tumour behaviour in the long-term.

PREDICTION OF HORMONE RESPONSE IN BREAST CANCER BY MICROARRAY ANALYSIS OF SEQUENTIAL TUMOUR BIOPSIES FROM PATIENTS RECEIVING NEOADJUVANT THERAPY WITH LETROZOLE.

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Background: Changes in tumour RNA expression may be monitored in sequential biopsies of individual tumours taken during neoadjuvant therapy and analysed by microarray analysis. In the present study, changes occurring within 10-14 days have been related to clinical response status assessed after 3 months of treatment.

Methods: 58 postmenopausal women with large operable ER-rich breast cancers were treated for 3 months with neoadjuvant letrozole. Clinical response was based on clinical and ultrasound changes. Cancers were sampled at diagnosis, 10-14 days and 3 months; RNA was extracted and hybridized on Affymetrix HG_U133A GeneChips.

Results: 52 cases were assessable for response; 37 (71%) responded (>50% reduction in tumour volume) and 15 were classified as minimal or no response. Changes in expression of 125 gene probes were informative in distinguishing between tumours subsequently displaying clinical response and those not. The gene onthology of the probe sets included protein metabolism (26%), transcription/translation (18%), signal transduction (14%), cell proliferation/apoptosis (14%). Clustering of these gene changes produced profiles highly predictive of response/resistance to letrozole.

Conclusions: Changes in pattern of gene expression can be detected in biopsies taken before and after 14 days treatment with neoadjuvant letrozole. These may elucidate the mechanisms of tumour response and allow early recognition of response/resistance. Patterns of expression changes can be used to predict subsequent tumour response to treatment.

Neoadjuvant letrozole is equally effective in Her 2 positive and negative breast cancers

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Her 2 status does not influence response to neoadjuvant letrozole.

Background: 69% of ER+ and Her 2 positive breast cancers responded to neoadjuvant letrozole, whereas only 17% of such tumours responded to tamoxifen in the study reported by Ellis et al (J Clin Oncol 2001:19:3808-3816). In ER+, Her 2 negative cancers the response rate to letrozole was 53%. This study used an antibody which is not currently in routine use for Her 2 testing and considered all 2+ and 3+ staining as overexpression. The current study set out to further investigate the interaction between Her 2 status and response to neoadjuvant letrozole.

Patients and Methods: 172 postmenopausal women with large operable or locally advanced ER rich (ER Allred score 6 or more) breast cancers were enrolled into a prospective audit assessing response to 3 months of neoadjuvant letrozole 2.5mg per day. Her 2 status was assessed using the Hercept test with FISH for 2+ samples. Response was assessed clinically and by ultrasound. Response rate and % reduction in tumour area and volume in Her 2 positive (3+ or 2+ and FISH positive) tumours have been compared with cancers classified as Her 2 negative (0,+ or 2+ and FISH negative).

Results: Of the 172 patients, 18 tumours were classified as Her 2 positive (either 3+ or 2+ and FISH +ve) and 154 were Her 2 negative.

Clinical Responses: At 3 months by WHO criteria 106/154 (69%) Her 2 negative and 11/18 (61%) Her 2 positive tumours had a clinical response p=0.506, Fisher's exact test. Details of tumour response are given in the table.

Reductions in tumour area and volume during letrozole treatment (volume calculated using the formula $d^3/6$).

	Her 2 Negative		Her 2 Positive	
	Median	95% CI	Median	95% CI
Clinical area	64%	57-68	64%	45-91
Clinical volume	78%	73-84	68	52-92
Ultrasound area	52%	48-60	47%	41-70
Ultrasound volume	67%	62-72	66%	37-83

None of the differences between Her 2 negative and Her 2 positive cancers were significant.

Conclusion: Neoadjuvant letrozole in this series of ER + breast cancers was equally effective in both Her 2 positive and negative tumours. It reduced tumour volume at 3 months by at least 66% in both groups. The efficacy of letrozole does not appear to be influenced by Her 2 status.